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Application Number	**09/710,058**
Filing Date	**November 10, 2000**
First Named Inventor	**ANDERSON, DAVID**
Group Art Unit	**1639**
Examiner Name	**Liu, Sue Xu**
Attorney Docket Number	**RIGL-011**

ENCLOSURES (check all that apply)

- [x] Credit Card Payment Form
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SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Signing Attorney/Agent (Reg. No.)	**JAMES S. KEDDIE, PH.D., 48,920** **BOZICEVIC, FIELD & FRANCIS, LLP**
Signature	James S. Keddie
Date	**March 8, 2007**

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APPEAL BRIEF

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Attorney Docket No.	RIGL-011
Confirmation No.	4112
First Named Inventor	ANDERSON, DAVID
Application Number	09/710,058
Filing Date	November 10, 2000
Group Art Unit	1639
Examiner Name	Sue Liu

Title: *"METHODS AND COMPOSITIONS COMPRISING RENILLA GFP"*

Sir:

This Brief is filed in support of Appellants' appeal of the rejections set forth in the Office Action dated December 13, 2006. A Notice of Appeal was filed on January 12, 2007.

The Commissioner is hereby authorized to charge deposit account number 50-0815 to cover any fee required for filing this brief.

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REAL PARTY IN INTEREST

The real party in interest in this case is Rigel Pharmaceuticals, Inc.

RELATED APPEALS AND INTERFERENCES

There are no appeals pending which would directly affect, be directly affected by, or have a bearing on the Board's decision in the instant appeal.

STATUS OF CLAIMS

Claims 1-3 and 20-22 are pending.

Claims 1-3 and 20-22 are appealed.

Claims 4-19 are cancelled.

STATUS OF AMENDMENTS

No amendments to the claims were filed subsequent to issuance of the prior Office Action. All amendments have been entered.

SUMMARY OF CLAIMED SUBJECT MATTER

The claimed subject matter relates to retroviral vectors that contain a polynucleotide that encodes a green fluorescent protein (GFP) having the amino acid sequence of SEQ ID NO:2. SEQ ID NO:2 sets forth the amino acid sequence of the wild-type *Renilla* GFP. Thus, the claims relate to a retroviral vector that encodes a wild-type *Renilla* GFP.

A description of each of the appealed claim follows below.

Claim 1 recites a retroviral vector (page 23, lines 28-33) comprising a polynucleotide encoding a green fluorescent protein (GFP; page 2, lines 24-25) having the amino acid sequence of SEQ ID NO:2 (page 3, lines 10-13), where SEQ ID NO:2 sets forth the amino acid sequence of the wild-type *Renilla mulleri* GFP (page 3, lines 10-13; Fig. 2).

Claim 2 recites a retroviral vector (page 23, lines 28-33) comprising a first encoding polynucleotide, an IRES site (page 3, lines 2-4), and a polynucleotide encoding a green fluorescent protein (GFP) having the amino acid sequence of SEQ ID NO:2.

Claim 3 recites a mammalian cell (page 25, lines 17-25) comprising the retroviral vector of claims 1 or 2.

Claim 20 recites a retroviral vector of claim 1 or 2, wherein said polynucleotide comprises a human codon-optimized nucleic acid encoding a *Renilla* GFP (page 70, lines 3-5).

Claim 21 recites a mammalian cell (page 25, lines 17-25) comprising: a retroviral vector comprising a polynucleotide encoding a green fluorescent protein (GFP) having the amino acid sequence of SEQ ID NO:2; wherein fluorescence of said GFP can be detected by fluorescence-activated cell sorting (FACS; page 56, lines 17-25).

Claim 22 recites a mammalian cell (page 25, lines 17-25) comprising: a retroviral vector comprising a polynucleotide encoding a green fluorescent protein (GFP) having the amino acid sequence of SEQ ID NO:2; wherein said mammalian cell is in the presence of a test agent (page 34, lines 7-8), and wherein an effect of said test agent is detectable by detecting fluorescence of said GFP (page 46, lines 21-29).

GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

1. Whether claims 1-3 and 20 are unpatentable under 35 U.S.C. § 103 over Bryan (U.S. Patent 6,232,107) in view of Aran (Cancer Gene Therapy 1998 5:195-206).

2. Whether claim 20 is unpatentable under 35 U.S.C. § 103 over Bryan in view of Aran or Zolutukhin (U.S. Patent 5,874,304).

3. Whether claims 1-3 and 20 are unpatentable under 35 U.S.C. § 103 over Zolutukhin in view of Bryan.

4. Whether claims 1, 3 and 20-22 are unpatentable under 35 U.S.C. § 103 over Bierhuizen (Biochem. Biophys. Res. Comm. 1997 234:371-375) in view of Bryan.

5. Whether claims 1-3 and 20-22 are unpatentable under 35 U.S.C. § 103 over Bierhuizen in view of Bryan and Aran.

6. Whether claims 1, 3 and 20-22 are unpatentable under 35 U.S.C. § 103 over Anderson (PNAS 1996 93: 8508-8511) in view of Bryan.

ARGUMENTS

Arguments directed to all rejections

As noted above, the claims at issue in this case recite a retroviral vector that encodes a wild-type *Renilla* green fluorescent protein (GFP).

Claim 1 is illustrative of all of the appealed claims:

1. A retroviral vector comprising a polynucleotide encoding a green fluorescent protein (GFP) having the amino acid sequence of SEQ ID NO:2.

All of the pending claims are rejected as obvious over Bryan (who discloses a wild-type *Renilla* GFP sequence), in combination with one or more secondary references that allegedly fill the void between Bryan's disclosure and what is being claimed.

In summary, Appellants submit that the rejected claims are patentable over Bryan and the secondary references because the state of the art at the time of filing[1] indicates that retroviral vectors that encode wild-type GFPs are or would be inoperative and therefore useless. Given this fact, the Appellants believe that one of skill in the art would not practice the claimed vector with any reasonable expectation of success. In other words, Appellants submit that cannot be obvious to make something that would be expected to be inoperative and therefore useless. Since there is no reasonable expectation of success, a *prima facie* case of obviousness cannot exist, and this rejection should be reversed.

The Appellants believe that in this case a patent is warranted because they found success in an area in which all others report only failure, namely the expression of a wild type GFP using a retroviral vector.

The main point that Appellants wish to convey to the Board in this Appeal Brief is set

[1] See the Aran, Hanazono, Levy, Cheng and Anderson references enclosed in the Exhibits section of this Appeal Brief. All of these references are of record, and are discussed in greater detail below.

forth above. To the extent that further discussion is deemed necessary, the Board is respectfully referred to the following.

In *Graham v. John Deere* (1966), the Supreme Court held that in making a determination of obviousness under 35 U.S.C. § 103 the courts and the Patent and Trademark Office should make several basic factual inquiries. One of the required inquiries is "the scope and content of the prior art".[2]

Further, the case law is replete with decisions that recognize that an invention that otherwise might be viewed as an obvious modification of something known in the prior art will not be deemed obvious in a patent law sense because of uncertainty in the art or the absence of a reasonable probability of success.[3] In other decisions, nonobviousness and patentability are found when one or more prior art references "teach away" from the invention.[4] Disclosures that diverge from and teach away from the invention cannot be disregarded.[5]

In particular, the Supreme Court stated in *United State v. Adams* that "known disadvantages in old devices which would naturally discourage the search for new inventions

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[2] *Graham v. John Deere*, 383 U.S. 1, 17, 148 USPQ 459 (1966).

[3] *See, e.g. Boehringer Ingelheim Vetmedica, Inc. v. Schering-Plough Corp.*, 320 F.3d 1339, 1354, 65 USPQ2d 1961 (Fed. Cir. 2003) ("there can be little better evidence negating an expectation of success than actual reports of failure. See, e.g., In re Rinehart, 531 F.2d 1048, 1053-54, 189 USPQ 143, 148-49 (CCPA 1976).")"); *Ortho Pharmaceutical Corp. v. Smith*, 959 F.2d 936, 943, 22 USPQ2d 1119, 1125 (Fed. Cir. 1992); *Hybritech Inc. v. Monoclonal Antibodies, Inc.* 802 F.2d 1367, 231 USPQ 81 (Fed. Cir. 1986); *Merck & Co. v. Danbury Pharmacal Inc.*, 694 F. Supp. 1, 29, 8 USPQ2d 1793, 1816 (D. Del. 1988), aff'd, 873 F.2d 1418, 10 USPQ2d 1682 (Fed. Cir. 1989).

[4] *See, e.g Gillette Co. v. S.C. Johnson & Sons, Inc.*, 919 F.2d 720, 724, 16 USPQ2d 1923, 1927 (Fed. Cir. 1990) (the closest prior art reference "would likely discourage the art worker from attempting the substitution suggested by [the inventor/patentee]."). See also *Singh v. Brake*, 317 F.3d 1334, 1346, 65 USPQ2d 1641 (Fed. Cir. 2003) ("whether or not a reference 'teaches away' from a claimed invention" is "relevant in determining whether or not a claimed invention would have been obvious"); *In re Peterson*, 315 F.3d 1325, 1331, 65 USPQ2d 1379 (Fed. Cir. 2003) ("an applicant may rebut a prima facie case of obviousness by showing that the prior art teaches away from the claimed invention in any material respect."); *McGinley v. Franklin Sports, Inc.*, 262 F.3d 1339, 1354, 60 USPQ2d 1001 (Fed. Cir. 2001) If references taken in combination would produce a 'seemingly inoperative device,' we have held that such references teach away from the combination and thus cannot serve as predicates for a prima facie case of obviousness. *In re Sponnoble*, 405 F.2d 578, 587, 160 USPQ 237, 244, 56 C.C.P.A. 823 (1969) (references teach away from combination if combination produces "seemingly inoperative device"); see also *In re Gordon*, 733 F.2d 900, 902, 221 USPQ 1125, 1127 (Fed. Cir. 1984) (inoperable modification teaches away)."); *In re Haruna*, 249 F.3d 1327, 1335, 58 USQP2d 1517 (Fed. Cir. 2001) ("A reference may be said to teach away when a person of ordinary skill, upon reading the reference, ... would be led in a direction divergent from the path that was taken by the applicant.' *Tec Air, Inc. v. Denso Mfg. Mich. Inc.*, 192 F.3d 1353, 1360, 52 USPQ2d 1294, 1298 (Fed. Cir. 1999)."); *Mentor H/S, Inc. v. Medical Device Alliance, Inc.*, 244 F.3d 1365, 58 USPQ2d 1321, 1328 (Fed. Cir. 2001).

[5] *Dow Chemical Co. v. United States*, 20 Cl. Ct. 623, 630, 18 USPQ2d 1657, 1662 (Cl. Ct. 1990).

may be taken into account in determining obviousness"[6]. Likewise in *In re Sponnoble* the court stated that if a combination of references would produce a "seemingly inoperative device,"[7] then they should not be combined.

All of the pending claims are rejected as obvious over Bryan (which discloses a wild-type *Renilla* GFP sequence), in combination with one or more secondary references that allegedly fill the void between Bryan's disclosure and what is being claimed. Although each rejection is different, the general thrust of each rejection is the same: Bryan's wild-type *Renilla* GFP, in combination with the retroviral vector of a secondary reference, renders the instant claims obvious. The Examiner argues that one of skill in the art would be motivated to make and use the claimed subject matter because the spectral properties of wild-type *Renilla* GFP were well known to be superior to the spectral properties of wild-type *Aequoria* GFP - the "industry standard" GFP in the mid to late 1990's.

The Appellants agree with the Examiner in that the amino acid sequence of wild-type *Renilla* GFP was known prior to filing of their patent application. The Appellants also agree that retroviral vectors containing altered *Aequoria* GFP were well known and had been successfully used prior to filing their patent application. Finally, the Appellants agree that the superior spectral properties of wild-type *Renilla* GFP were well known prior to filing their patent application.

Notwithstanding the above, the Appellants believe that the facts of this case logically lead to the conclusion that, at the time of filing, there would have been no reasonable expectation that the claimed retroviral vector containing a wild type *Renilla* GFP would work. In view of this conclusion and applying current law, the claimed retroviral vectors should be patentable.

Evidence that there is no reasonable expectation that the claimed retroviral vector would work is found in five different references (Aran, Hanazono, Levy, Cheng and Anderson[8]; the "supporting references") each of which unequivocally state that retroviral vectors encoding wild-type GFPs do not work. The supporting references were well known

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[6] *United States v. Adams* 383 U.S. 39, 52, 148 USPQ 479, 484 (1966).

[7] *In re Sponnoble* 405 F.2d 578, 587, 160 USPQ 237, 244, 56 C.C.P.A. 823 (1969)

[8] All references are of record. Aran and Anderson are cited in rejections under 35 U.S.C. § 103.

and available at the time of filing and, as such, indicate the state of the art at the time of filing. The supporting references are supplied herewith in the Evidence Appendix.

In particular, the statements in the supporting references can be summarized as follows:

- Aran states that wild type GFP fluorescence was "undetectable";[9]
- Hanzano states that their attempts to isolating wild-type GFP-expressing lines "failed";[10]
- Levy states that "wildtype GFP could never be visualized";[11]
- Cheng states the wild type GFP expression "failed";[12] and
- Andersen states that fluorescence was "not sufficient to resolve infected from uninfected cells".[13]

These statements are unequivocal, and represent fair evidence that the prospects of a retroviral vector encoding other wild-type GFPs would be quite gloomy.

Further, the landmark publications of Levy and Cheng showed that successful expression of GFP using a retroviral vector *required* altering the amino acid sequence of the GFP. As such, these references further *teach away* from what is being claimed.

The references show that at the time of filing there would be no reasonable expectation that the claimed retroviral vector would work. Using the language of the court in *In re Sponnoble*, *supra*, the combination of references proposed in this Office Action would

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[9] See the first full paragraph of page 204 of Aran's disclosure, where Aran states that when a retroviral vector encoding a wild type *Aequorea* GFP was introduced into in a mammalian cell, fluorescence was "***undetectable***".

[10] Hanazono (Hum. Gene Ther. 1997, 8:1313-9) who stated in the abstract that "many attempts by our laboratory to isolate stable retroviral producer cell clones secreting biologically active vectors containing either the highly fluorescent S65T-GFP mutant or humanized GFP have ***failed***", and with reference to retroviral vectors encoding GFP, stated in the overview "***stable clones produced neither virus nor GFP***" and "GFP may not be a suitable selective marker in mammalian gene transfer systems".

[11] Levy et al. (Nature Biotechnology 1996, 14: 610-4, at p. 613, first full paragraph) who states that "Our experiments are in agreement with these results in that transient transfection which transfers multiple transgene copies of wildtype GFP expression cassettes were visualized, but we found that stable transduced lines with a single transgene copy of ***wildtype GFP could never be visualized*** by fluorescence microscopy (Table 1)".

[12] Cheng et al (Nature Biotechnology 1996, 14: 606-609) who states in the second paragraph of the introduction "the expression and detection of wildtype GFP (wtGFP) in mammalian cells reportedly ***failed***".

[13] Anderson et al (Proc. Natl. Acad. Sci. 1996 93: 8505-8511; of record and cited in this Office Action) states in the background that suboptimal excitation spectra of wild type GFP "*precludes the detection of wtGFP* when a single copy of the gene is stably integrated", and in the first paragraph of the results section, with reference to a population of cells infected with a retroviral vector encoding wild type *Aequoria* GFP, states: "the difference in *fluorescence was not sufficient* to resolve infected from uninfected cells".

produce a retroviral vector that would be "seemingly inoperative". Further, since the references teach that only non-wild type GFP can be expressed using a retroviral vector, the references teach away from what is being claimed.

Applying current case law to this fact pattern, the claimed retroviral vectors should be patentable.

In the Office Action, the Examiner attempts to undermine the Appellants' position by arguing that the Appellants' "reasonable expectation of success" arguments are directed to the *use* of the claimed vector, rather than the *making* of the claimed vector.

In response, the Appellants submit that the caselaw is very clear that evaluating the expected *operability* of the device produced by the suggested combination of references is critical to determining non-obviousness.[14] In other words, the Examiner may argue that any molecular biologist would have a reasonable expectation of success in making the claimed vector. The question here, however, is not whether one of skill in the art could *make* the claimed vector with a reasonable expectation of success. Rather the question is whether one of skill in the art would *use* the claimed vector with a reasonable expectation of success. If there is no reasonable expectation of success to use the claimed vector, then there would be no motivation to make the vector in the first place.

Since one of skill in the art would not be able to *use* the claimed vector with a reasonable expectation of success, the Appellants prior arguments still stand with equal force.

In the Office Action, the Examiner further attempts to undermine the Appellants' position by arguing that the statements made in the references that support the Appellant's position (i.e., the "supporting references") are irrelevant because they are directed to only wild-type *Aequoria* GFP and not wild-type *Renilla* GFP.

The Appellants acknowledge that *Renilla* GFP is not explicitly mentioned in any of the supporting references. However, it would be readily apparent that one of skill in the art, in

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[14] *McGinley v. Franklin Sports, Inc.*, 262 F.3d 1339, 1354, 60 USPQ2d 1001 (Fed. Cir. 2001) If references taken in combination would produce a 'seemingly inoperative device,' we have held that such references teach away from the combination and thus cannot serve as predicates for a prima facie case of obviousness. *In re Sponnoble*, 405 F.2d 578, 587, 160 USPQ 237, 244, 56 C.C.P.A. 823 (1969) (references teach away from combination if combination produces seemingly inoperative device); see also *In re Gordon*, 733 F.2d 900, 902,

evaluating their chances of success in expressing a wild-type *Renilla* GFP using a retroviral vector, would logically look towards the closest prior art for guidance, rather than ignore the closest prior art. Since there is adequate evidence that wild type *Aequoria* GFP cannot be expressed using a retroviral vector, one of skill in the art would believe the same would very likely be true for a wild type *Renilla* GFP.

If the Examiner deems that references directed to retroviral vectors encoding *Aequoria* GFP are simply not relevant to claims directed to a retroviral vector encoding *Renilla* GFP, then such references should not be citable to render obvious claims directed to that very same subject matter.

Giving full weight to references relating to *Aequoria* GFP to support a rejection of claims directed to a *Renilla* GFP, while, at the same time, ignoring the teachings of those references to undermine the Appellants' position seems inconsistent. Either the teachings of these references should be given full weight in supporting the Appellants' arguments, or the rejection should be withdrawn as being based on references that are not relevant to the claimed subject matter. Either way, the rejections should be withdrawn.

The Examiner seems to suggest that because the supporting references do not explicitly state "don't express *Renilla* GFP using a retroviral vector – it won't work", the teachings of the supporting references are not relevant. The Appellants submit, however, that the teachings of those references show that retroviral vectors encoding wild-type GFPs is at best highly unpredictable and more likely impossible. The teachings of those references are relevant the patentability of the rejected claims and, as such, should not be ignored.

In summary, the Appellants submit that the disclosures of Aran, Hanazono, Levy, Cheng and Anderson represent fair evidence that one of skill in the art would have no reasonable expectation of success in expressing a wild-type *Renilla* GFP using a retroviral vector. As such, the claimed retroviral vectors should be patentable, and all of these rejections should be reversed.

All rejections are addressed individually below.

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221 USPQ 1125, 1127 (Fed. Cir. 1984) (inoperable modification teaches away).

Rejection under 35 U.S.C. § 103 - Bryan and Aran

Claims 1-3 and 20 remain rejected under 35 U.S.C. § 103(a) as being unpatentable over Bryan and Aran. The Office Asserts that Bryan's GFP, in combination with Aran's retroviral vectors, renders the subject matter of the instant claims obvious.

In view of the general discussion set forth above, the Appellants submit that there would no reasonable expectation of success in using the claimed subject matter at the time of filing of the instant application. As such, this rejection may be reversed.

The Appellants particularly note that the first full paragraph of page 204 of Aran's disclosure states that when a retroviral vector encoding a wild type *Aequoria* GFP was introduced into in a mammalian cell, fluorescence was "***undetectable***". As such, Aran's disclosure, itself, teaches that the combination of Bryan and Aran would produce "a seemingly inoperative" vector.[13]

Reversal of this rejection is respectfully requested.

Rejection under 35 U.S.C. § 103 - Aran, Bryan and Zolutukhin

Claim 20 is rejected under 35 U.S.C. § 103(a) as being unpatentable over Aran, Bryan and Zolutukhin. The Office Asserts that Aran's retroviral vectors, Bryan's Renilla GFP and Zolutukhin's human codon optimized GFP renders the subject matter of the instant claims obvious.

In view of the general discussion set forth above, the Appellants submit that there would no reasonable expectation of success in using the claimed subject matter at the time of filing of the instant application. As such, this rejection may be reversed.

The Appellants particularly note that the first full paragraph of page 204 of Aran's disclosure states that when a retroviral vector encoding a wild type *Aequoria* GFP was introduced into in a mammalian cell, fluorescence was "***undetectable***". As such, Aran's disclosure, itself, teaches that the combination of Bryan, Aran and Zolutukhin would produce "a seemingly inoperative" vector.[13]

Reversal of this rejection is respectfully requested.

Rejection under 35 U.S.C. § 103 - Zolutukhin and Bryan

Claims 1-3 and 20 are rejected under 35 U.S.C. § 103(a) as being unpatentable over Zolutukhin and Bryan. The Office Asserts that Zolutukhin's human codon optimized GFP retroviral vector, in combination with Bryan's *Renilla* GFP, renders the subject matter of the instant claims obvious.

In view of the general discussion set forth above, the Appellants submit that there would no reasonable expectation of success in using the claimed subject matter at the time of filing of the instant application. As such, this rejection may be reversed.

Reversal of this rejection is respectfully requested.

Rejection under 35 U.S.C. § 103 - Bierhuizen and Bryan

Claims 1-3 and 20-22 are rejected under 35 U.S.C. § 103(a) as being unpatentable over Bierhuizen (Biochem. Biophys. Res. Comm. 1997 234: 371-375) in view of Bryan. The Office Asserts that Bierhuizen's retroviral vectors, in combination with Bryan's GFP, renders the subject matter of the instant claims obvious.

In view of the general discussion set forth above, the Appellants submit that there would no reasonable expectation of success in using the claimed subject matter at the time of filing of the instant application. As such, this rejection may be reversed.

The Appellants submit that Bierhuizen does not undermine the Appellants' arguments because: a) Bierhuizen is a single reference in a field in which many others report repeated failure; and b) Bierhuizen, in fact, reports only marginal results their their vector.

In particular, Bierhuizen notes that at 24 hours after transduction, wild type GFP expression is extremely weak compared to cells expressing mutant GFP. Further, according to

the first col. of page 373, Bierhuizen's assays were performed on cells that had been transduced and then cultured for only *24 hours*. As such, Bierhuizen fails to report stable cell lines that express wild type *Aequoria* GFP. Given the teachings of Aran, Hanzano, Levy, Cheng and Anderson (cited above), such cell lines would be impossible to make. Bierhuizen's results therefore highlight the uncertainty in this field, and do not diminish the Appellants arguments.

Since Bierhuizen fails to report cells that stably express wild-type *Aequoria GFP* using a retroviral vector and also reports only very weak expression a short time after transduction, Bierhuizen's disclosure supports the Appellants' position that one of skill in the art would not practice the invention with a reasonable expectation of success.

Reversal of this rejection is requested.

Rejection under 35 U.S.C. § 103 – Bierhuizen in view of Bryan and Aran

Claims 1-3 and 20-22 are rejected under 35 U.S.C. § 103(a) as being unpatentable over Bierhuizen in view of Bryan and Aran.

The Office asserts that Bierhuizen's retroviral vectors, in combination with Bryan's GFP and Aran's IRES renders the subject matter of the instant claims obvious.

In view of the generally discussion set forth above, the Appellants submit that there would no reasonable expectation of success in using the claimed subject matter at the time of filing of the instant application. As such, this rejection may be reversed.

The Appellants particularly note that the first full paragraph of page 204 of Aran's disclosure states that when a retroviral vector encoding a wild type *Aequoria* GFP was introduced into in a mammalian cell, fluorescence was "***undetectable***". As such, Aran's disclosure, itself, teaches that the combination of Bryan and Aran would produce a seemingly inoperative vector.

As submitted above, however, Bierhuizen does not undermine the Appellants' arguments because: a) Bierhuizen is a single reference in a field in which many others report repeated failure; and b) Bierhuizen, in fact, reports only marginal results their vector.

Reversal of this rejection is requested.

Rejection under 35 U.S.C. § 103 – Anderson in view of Bryan

Claims 1-3 and 20-22 are rejected under 35 U.S.C. § 103(a) as being unpatentable over Anderson in view of Bryan.

The Office Asserts that Anderson's retroviral vectors, in combination with Bryan's GFP, renders the subject matter of the instant claims obvious.

In view of the generally discussion set forth above, the Appellants submit that there would no reasonable expectation of success in using the claimed subject matter at the time of filing of the instant application. As such, this rejection may be withdrawn.

The Appellants particularly note that Anderson states in the background section that suboptimal excitation spectra of wild type GFP "*precludes the detection of wtGFP* when a single copy of the gene is stably integrated", and in the first paragraph of the results section, with reference to a population of cells infected with a retroviral vector encoding wild type *Aequoria* GFP, states: "the difference in *fluorescence was not sufficient* to resolve infected from uninfected cells"

As such, Anderson's disclosure, itself, teaches that the combination of Anderson and Bryan would produce a seemingly inoperative vector.

SUMMARY

The Appellants submit that all current rejections lack merit and should be reversed.

RELIEF REQUESTED

The Appellants respectfully request that all of the rejections be reversed, and that the application be remanded to the Examiner with instructions to issue a Notice of Allowance.

Respectfully submitted,

Date: March 8, 2007

By: _____
James S. Keddie
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Claims Appendix

1. A retroviral vector comprising a polynucleotide encoding a green fluorescent protein (GFP) having the amino acid sequence of SEQ ID NO:2.

2. A retroviral vector comprising a first encoding polynucleotide, an IRES site, and a polynucleotide encoding a green fluorescent protein (GFP) having the amino acid sequence of SEQ ID NO:2.

3. A mammalian cell comprising a retroviral vector according to claim 1 or 2.

4-19. (Cancelled)

20. The retroviral vector of claim 1 or 2, wherein said polynucleotide comprises a human codon-optimized nucleic acid encoding a Renilla GFP.

21. A mammalian cell comprising:

a retroviral vector comprising a polynucleotide encoding a green fluorescent protein (GFP) having the amino acid sequence of SEQ ID NO:2;

wherein fluorescence of said GFP can be detected by fluorescence-activated cell sorting (FACS).

22. A mammalian cell comprising:

a retroviral vector comprising a polynucleotide encoding a green fluorescent protein (GFP) having the amino acid sequence of SEQ ID NO:2;

wherein said mammalian cell is in the presence of a test agent, and wherein an effect of said test agent is detectable by detecting fluorescence of said GFP.

EVIDENCE APPENDIX

No evidence submitted under 37 CFR §§ 1.130 or 1.131 has been relied upon by Appellants in this Appeal.

The following references are included in the Evidence Appendix of this Appeal Brief. All of these references are of record in the instant application.

- Aran (Cancer Gene Therapy 1998 5:195-206)
- Hanazono (Hum. Gene Ther. 1997, 8:1313-9)
- Levy et al. (Nature Biotechnology 1996, 14: 610-4, at p. 613).
- Cheng et al (Nature Biotechnology 1996, 14: 606-609)
- Anderson et al (Proc. Natl. Acad. Sci. 1996 93: 8505-8511

RELATED PROCEEDINGS APPENDIX

There are no decisions rendered by a court or the Board which would directly affect or be directly affected by, or have a bearing on the Board's decision in the instant appeal.

Construction and characterization of bicistronic retroviral vectors encoding the multidrug transporter and β-galactosidase or green fluorescent protein

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Multidrug resistance (MDR) can be conferred by overexpression of the adenosine triphosphate-driven multidrug transporter P-glycoprotein (Pgp) known as *MDR*1. Thus, two potential applications of the *MDR*1 gene that may be useful in gene therapy are the protection of bone marrow cells from the cytotoxic effects of chemotherapy regimens in cancer patients and its possible use as an *in vivo* selectable gene when linked to a therapeutic gene. In this study, we have designed two retroviral bicistronic expression vectors by linking the *MDR*1 gene to the reporters known as β-galactosidase and the red-shifted green fluorescent protein (GFP). We report the creation of stable producer cell lines that synthesize virus particles carrying the MDR-internal ribosomal entry site (IRES)-*lacZ* and the MDR-IRES-GFP transgenes. These transcriptional fusions allow coordinate expression of Pgp and the reporter gene product to easily mark the MDR phenotype. Using the MDR-IRES-*lacZ* retrovirus, we demonstrate that periodic pulses of cytotoxic drug selection with a Pgp substrate enable sustained, long-term expression of the reporter β-galactosidase in otherwise unstable transductants. We have also incorporated the improved features of GFP as a reporter gene into our MDR-IRES-GFP retrovirus. This vector allows rapid and specific identification of *MDR*1 gene transfer and expression in living cells either by fluorescence microscopy or by fluorescence-activated cell sorter analysis. These two MDR/reporter gene systems should be useful for *in vivo* studies, the evaluation of the potential of the *MDR*1 gene in gene therapy applications, and as a monitor of the selective efficacy of its MDR phenotype.

Key words: P-glycoprotein; β-galactosidase; GFP; retroviral vector.

The potential of chemotherapy for cancer treatment is often impaired in tumor cells by the acquisition of cross-resistance to a spectrum of chemotherapeutic drugs. One of the major factors contributing to this phenomenon, termed multidrug resistance (MDR), is the expression of P-glycoprotein (Pgp) in the plasma membrane of resistant cells. It is an adenosine triphosphate-driven efflux pump capable of extruding a wide variety of cytotoxic natural product drugs.[1,2] Although the structure and precise mechanism of action of this transporter is not known, a great deal of effort has been directed recently to develop and screen novel compounds capable of interacting with Pgp to reverse MDR and improve chemotherapy.[3,4] Furthermore, since the demonstration that transfer of the *MDR*1 gene encoding Pgp into drug sensitive cells was sufficient to confer the MDR phenotype, attention has focused on its potential application as a selectable gene for gene therapy both *in vitro* and *in vivo*.[5]

Recent *in vitro* studies using retroviral vectors as gene transfer systems have demonstrated the potential of the *MDR*1 gene as a selectable marker and have optimized the configuration of bicistronic units containing the *MDR*1 gene and a therapeutic gene to maximize expression levels of the corresponding gene products.[6–8] This has been achieved by synthesizing transcriptional fusions containing an internal ribosomal entry site (IRES) element from the encephalomyocarditis virus (ECMV), which allows efficient cap-independent translation of the downstream gene.

The creation of transgenic mice expressing the *MDR*1 gene and the demonstration that their bone marrow becomes resistant to lethal doses of cytotoxic drugs that are substrates of Pgp has suggested that the *MDR*1 gene could be useful as a selectable gene *in vivo*.[9,10] Recent reports supporting this possibility have shown selection *in vivo* with Taxol of mouse bone marrow cells transduced *in vitro* with an *MDR*1 retrovirus.[11–13]

To facilitate investigation of the usefulness of the *MDR*1 gene as an *in vivo* selectable gene and the potential of the *MDR*1-based bicistronic vectors to carry-out and express a therapeutic gene of interest *in vivo*, it

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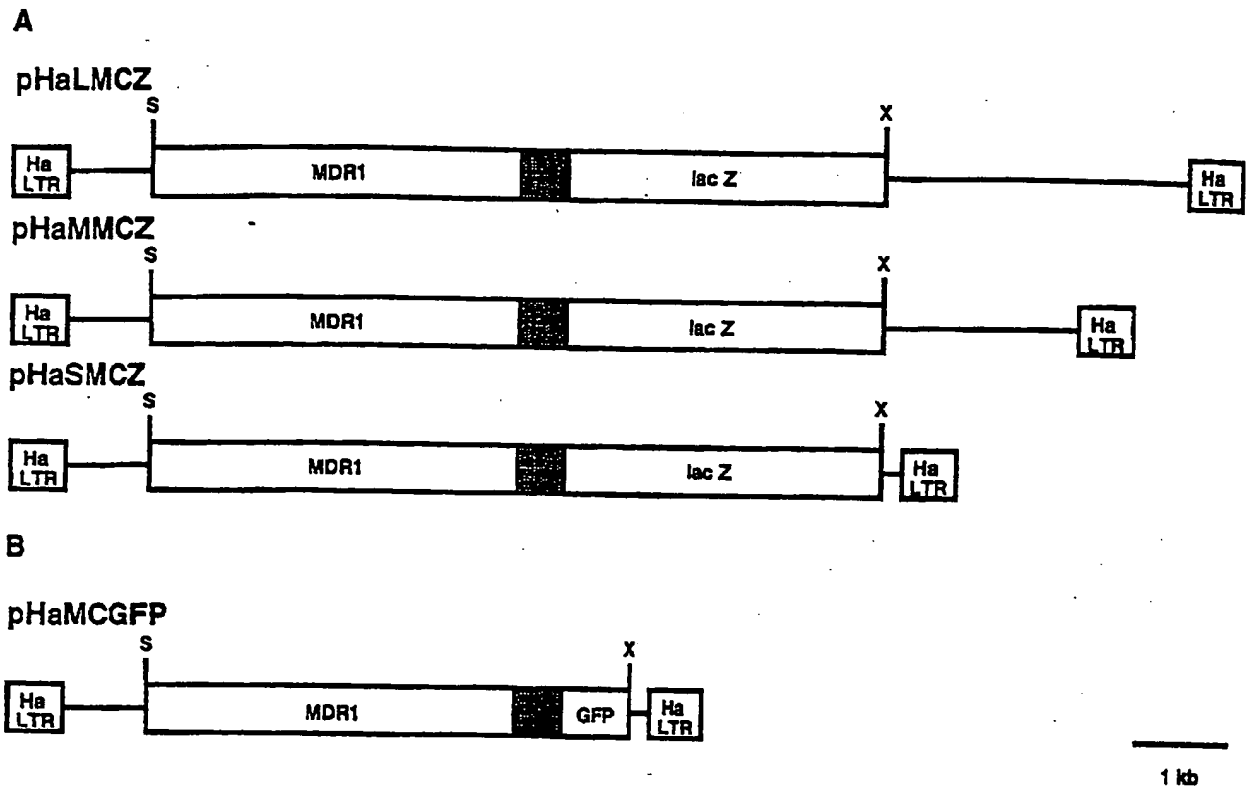

Figure 1. Proviral structure of the Harvey murine sarcoma-derived retroviral vectors pHaLMCZ, pHaMMCZ, pHaSMCZ, and pHaMCGFP. The bicistronic expression vectors pHaLMCZ, pHaMMCZ, and pHaSMCZ incorporate the MDR-IRES-*lacZ* transgene (A), and pHaMCGFP carries the MDR-IRES-GFP transgene (B). Gray boxes indicate the 503-bp ECMV IRES. HaLTR, Harvey murine sarcoma virus LTRs; MDR1, *MDR1* cDNA encoding human Pgp; *lacZ*, *E. coli lacZ* gene encoding the bacterial β-gal; GFP, GFP cDNA encoding a red-shifted, humanized GFP from *A. victoria*. S, *SstII*; X, *XhoI*.

would be very useful to have a quick, easy, and sensitive method to measure the expression of the nonselected transferred gene and the effects of drug selection schemes on the expression of the passenger gene.

In this study, we have designed two bicistronic units containing the *MDR1* gene and the reporter genes coding for *Escherichia coli* β-galactosidase (β-gal) (*lacZ*) and for a humanized, red-shifted green fluorescent protein (GFP) from the jellyfish *Aequorea victoria*.[14] We demonstrate that producer lines synthesizing MDR-IRES-*lacZ* and MDR-IRES-GFP virus particles are capable of transferring the MDR phenotype to recipient cells, which can be quickly and easily analyzed at the single-cell level by either measuring β-gal expression by histochemistry or GFP expression by fluorescence.

MATERIALS AND METHODS

Retroviral vector constructions

The synthesis of a bicistronic unit containing the MDR-IRES-*lacZ* transcriptional fusion was carried out as follows: from the pCH110 expression vector (Pharmacia, Uppsala, Sweden), containing the *E. coli* β-gal gene, we amplified a 450-base pair (bp) fragment by polymerase chain reaction (PCR); the fragment extended from the methionine (ATG) initiation codon of the *E. coli* β-gal DNA to its unique *Bsu36I* site. In the forward 31 oligomer (mer) primer, 5'-GCTGGCCATGGGC GAAAAATACATCGTCACC-3', we engineered an *NcoI* site containing the ATG codon of β-gal. The 32-mer reverse primer, 5'-CATATG CATATGCGGCCTCAGGAAGAT CGCAC-3', included *NdeI* and *Bsu36I* restriction sites. The amplified β-gal fragment was *NcoI/NdeI* restricted and introduced into the pCITE2a(+) vector (Novagen, Madison, Wis), which contains the 510-bp fragment from the ECMV 5' untranslated region (IRES element), creating the pCPCRZ vector. Once in this vector, the β-gal PCR fragment was sequenced in both directions to verify that no mistakes were introduced in the PCR process. Next, the remaining β-gal DNA fragment was excised from the pCH110 vector by restriction with *Bsu36I/DraI* and ligated to the *Bsu36I/EcoRV* fragment from pCPCRZ. The generated pCZ vector contained the IRES element linked to the complete *lacZ* gene by an *NcoI* site. Finally, pCZ was digested with *AseI* and blunt-ended. The isolated 4-kilobase (kb) IRES-*lacZ* fragment was then assembled downstream to the *MDR1* gene as previously described.[6] The resulting pMCZ vector, with the complete MDR-IRES-*lacZ* cassette in the correct configuration, was excised by *SstII/XhoI* restriction and assembled into the different Harvey murine sarcoma virus-based retroviral backbones (Fig 1).

Both, "pHaM" and "pHaS" were derived from the original retroviral backbone, "pHaL," included in the Harvey murine sarcoma virus expression vector pCO1.[15] pHaM was obtained by replacing 1071-bp sequences expanding from the *XhoI* to the *BstEII* unique sites of the pHaL backbone for an *XhoI-NotI-BstEII* adaptor. The short, optimized pHaS backbone was derived from the pHaMA vector.[16]

This pHaS backbone was also used for the construction of pHaMCGFP, carrying a bicistronic MDR-IRES-GFP cassette that was cloned as follows: from the pGreen Lantern-1 expression vector (Life Technologies, Grand Island, NY), containing a red-shifted variant of the GFP cDNA,[14] we amplified a 166-bp fragment by PCR; the fragment expanded from the ATG initiation codon of GFP to its *NcoI* site. In the forward 40-mer primer, JA-52 (5'-GATGCTGGCCATGGGCAAGG GCGAGGAACTGTTCACTG GC-3') we engineered an *NcoI* site containing the ATG starting codon for GFP. The 25-mer reverse primer JA-53 (5'-CAGTGTTGGCCATGGCACAGG GAGC-3') included the internal *NcoI* site of GFP. The remaining GFP cDNA was excised from the pGreen Lantern-1 vector by *NcoI/NotI* restriction and ligated to the equally restricted pCITE2a(+) (Novagen). This new pCTGFP vector was restricted with *NcoI*, dephosphorylated, and ligated to the previously generated, *NcoI/NcoI*-restricted, PCR fragment to create pCGFP which was verified by restriction analysis and sequencing. The pCGFP vector included the IRES element linked to the complete GFP cDNA by an *NcoI* site. Finally, pCGFP was digested with *AseI* and blunt-ended. The isolated 5.5-kb MDR-IRES-GFP fragment was then assembled downstream of the *MDR1* gene as previously described for the MDR-IRES-*lacZ* vector series and introduced into the optimized pHaS retroviral backbone to create pHaMCGFP.

Cell lines

NIH3T3 cells and the packaging cell lines PA317,[17] and GP+E86[18] were supplemented with 10% fetal calf serum (Life Technologies). ψ-CRE cells[19] were supplemented with 10% calf serum (Colorado Serum Company, Denver, Colo). All cells were maintained in Dulbecco's modified Eagle's medium (Quality Biologicals, Gaithersburg, Md) as previously described.[7]

Generation of ecotropic and amphotropic producer lines

To generate producer cell lines capable of synthesizing virus particles containing the complete MDR-IRES-*lacZ* bicistronic cassette, we first transfected ψ-CRE-packaging cells with 10 μg of retroviral vector DNA by calcium phosphate coprecipitation.[6] Successfully transfected cells were selected with 30 ng/mL vincristine. Drug-resistant cell pools were subsequently expanded, and titers of the resulting drug-free supernatants were assayed by transducing NIH3T3 target cells. Successfully transduced cells were scored by either drug resistance by methylene blue staining[7] or β-gal activity by X-gal staining using the β-gal staining kit (Invitrogen, San Diego, Calif). To obtain both GP+E86 ecotropic and PA317 amphotropic producers, these cells were transduced with supernatants from the ψ-CRE producer pool and analyzed as described above. To obtain producer cells synthesizing the MDR-IRES-GFP retrovirus, we transfected PA317-packaging cells directly and selected them with 30 ng/mL vincristine. The resulting drug-resistant producer pool was titered by transducing NIH3T3 cells as previously described for the MDR-IRES-*lacZ* retrovirus.

RNA isolation and Northern blot analysis

Total RNA from transduced NIH3T3 clones was obtained using the RNeasy kit (Qiagen, Chatsworth, Calif) and analyzed as previously described,[20] using the MDR-specific HaM (B/N) probe and the *lacZ*-specific GAL (B/D) probe, a 2897-bp *Bsu36I/DraI* fragment from the *lacZ* gene.

Cell extraction and Western blot analysis

pHaSMCZ-transduced clones were resuspended in extraction buffer (60 mM potassium phosphate, pH 5.9, plus 0.1% Triton X-100) and lysed by a freeze-thaw cycle plus sonication, as previously described.[6] Cell extracts were resolved in 4–12 or 8–16% precast gradient polyacrylamide gels (Novex, San Diego, Calif), transferred to a nitrocellulose membrane (BA83, Schleicher & Schuell, Keene, NH), and incubated simultaneously with anti-Pgp C219 monoclonal antibody (mAb)[21] at a 1:1000 dilution and anti-β-gal mAb (Promega, Madison, Wis) at a 1:4000 dilution. A goat anti-mouse IgG conjugated with horseradish peroxidase (Amersham, Arlington Heights, Ill) at a 1:500 dilution was used as secondary antibody, as detected by chemiluminescence (Enhanced Chemiluminescence kit, Amersham).

Protein concentrations were determined by the bicinchoninic acid assay (Pierce, Rockford, Ill), using bovine serum albumin as a standard.

Fluorescence analysis

GFP-positive cells were scored either by fluorescence microscopy using a Zeiss Axiovert 35 (Carl Zeiss Inc., Thornwood, NY) equipped with a standard fluorescein filter set, or by flow cytometry, in which cells were trypsinized, pelleted, resuspended in PBS + 0.1% bovine serum albumin, and analyzed directly in a FACSort (Becton Dickinson, Mountain View, Calif).

Enzyme-linked immunosorbent assay (ELISA) determination of β-gal expression

For specific and quantitative determination of *E. coli* β-gal expression from pHaSMCZ-transduced cells, we used a sensitive colorimetric immunoassay (Boehringer Mannheim, Indianapolis, Ind), which was performed according to the manufacturer's instructions.

RESULTS

β-gal-expressing producer cell lines are generated only with the pHaSMCZ vector

The proviral structure of three retroviral vectors included in this study is illustrated in Figure 1. These vectors were designed to express bicistronic transcripts containing the *MDR1* gene as a selectable marker and the *E. coli* β-gal gene as a reporter gene. Translation of the second cDNA, the reporter β-gal, is cap-independent and driven by an IRES from the ECMV.

We anticipated that the packaging of the long bicistronic MDR-IRES-*lacZ* transcript (8 kb) into recombinant virus particles could be inefficient in the

original pHaLMCZ vector due to both the size limitations of retroviral vectors to ~8 kb of recombinant sequences[22,23] and the use of the original Harvey murine sarcoma retroviral backbone (Fig 1A),[15] which included >3 kb of rat-derived VL30 sequences before the 3′ long terminal repeat (LTR).

Therefore, to accommodate the MDR-IRES-*lacZ* bicistronic expression cassette in the retroviral vector without compromising the production of recombinant virus particles and/or the expression levels of both transgenes, we synthesized two new vectors: pHaMMCZ and pHaSMCZ. These vectors included modified retroviral backbones generated by deletion of part (pHaM, this work), or most (pHaS) of the rat-derived VL30 sequences[16] (see *Materials and Methods* for details). Transfection of these three vectors together with the control pHaMDR1/A vector into NIH3T3 cells and ψ-CRE packaging cells and selection in 30 ng/mL vincristine yielded drug-resistant colonies in all cases. The transfection efficiency for pHaLMCZ, pHaMMCZ, and pHaSMCZ was similar and slightly (2-3-fold) reduced compared with the pHaMDR1/A control vector. Moreover, X-gal staining of the drug-resistant cells revealed that 80–85% were β-gal positive.

To test whether ecotropic ψ-CRE packaging cells were capable of producing recombinant virus particles containing the MDR-IRES-*lacZ* transcriptional fusion, we took undiluted cell culture supernatants from ψ-CRE cells transfected with the three retroviral vectors and tested their transduction capability using NIH3T3 cells as targets. Only the pHaSMCZ vector, containing the smallest retroviral backbone, was capable of transducing the NIH3T3 cells, although titers were $<10^2$ colony-forming units (cfu)/mL. This vector conferred both the MDR phenotype and β-gal expression. This result confirms that size limitation is an important factor for packaging long transgenes into recombinant virus particles when using retroviral vectors.

We attempted to obtain high-titer ecotropic and amphotropic producers by superinfecting GP+E86 and PA317 packaging cells with five rounds of exposure to undiluted supernatants from the low-titer ψ-CRE producer pool. After intensive screening of single-cell producer clones, we could only reach titers of $0.5–1.0 \times 10^4$ drug-resistant colonies/mL for both ecotropic and amphotropic producers. A single-cell PA317 producer clone, C-6, with a titer of 1.0×10^4 cfu/mL, was chosen for all subsequent experiments. Consistent with other reports,[24,25] we also found heterogeneity in the pattern of X-gal staining on cells transduced with the pHaSMCZ producers (Table 1), even within cells arising from a successfully transduced single-cell clone (data not shown). At least part of this variability has been attributed to epigenetic turn off of β-gal expression. Moreover, among the drug-resistant transductants, a small proportion (10–15%) was negative for X-gal staining (Table 1). Northern blot analysis of some of these clones revealed a shortened transcript consistent with deletion of the β-gal sequence (see Fig 2, clone 3A-II).

Table 1. Histochemical Staining of pHaSMCZ-Transduced Cells

	Colony counts			
	Methylene blue staining	X-gal staining		
		(+)	(+/−)	(−)
Experiment 1	453	16	385	52
Experiment 2	340	20	270	50

For each experiment, one dish of pHaSMCZ-transduced NIH3T3 cells was split 1:6 and selected with 30 ng/ml vincristine at 2 days post-transduction. After 10 days of selection, two of the dishes were histochemically stained with methylene blue (for drug resistance) or X-gal (for β-gal activity) as described in *Materials and Methods*. After staining, plates were viewed at ×40 magnification. For X-gal staining, (+) refers to clones with more than 90% blue-stained cells, and (+/−) refers to clones with 5 to 90% blue-stained cells.

*Retrovirus-mediated transfer of the MDR-IRES-*lacZ *cassette into NIH3T3 cells*

To check the capability of the amphotropic C-6 producer clone to transfer and express the complete MDR-IRES-*lacZ* bicistronic unit in target cells, we transduced NIH3T3 cells at a multiplicity of infection of $\sim 10^{-1}$. Single-cell clones were obtained by limiting dilution of the corresponding pHaSMCZ-transduced cell pool. Some of these clones were tested by Northern blot analysis. Figure 2A shows the expression levels of the MDR-IRES-*lacZ* bicistronic transcript in drug-selected (2A-I, 6B-I, 6B-II, 1D-II), and unselected (2B-U, 4B-U, 1A-U) clones using the MDR-specific HaM (B/N) probe.[20] The same result was obtained using the *lacZ*-specific probe GAL (B/D) (data not shown). Clone 3A-II, which was drug-resistant but negative for β-gal expression by X-gal staining, was found deleted in the *lacZ* gene region. Thus, the amphotropic producer clone C-6 is capable of efficient transfer of a functional bicistronic MDR-IRES-*lacZ* cassette into the recipient cells.

We determined the Pgp and β-gal expression levels by immunoblotting cell extracts from the same clones analyzed by Northern blotting. Figure 2B shows that all clones express a broad 170-kDa band corresponding to glycosylated Pgp and a sharp 120-kDa band from soluble β-gal. As negative controls, we used both clone 3A-II, which was negative for β-gal expression as previously discussed, and untransduced NIH3T3 cells. The weak Pgp band detected in the NIH3T3 cells is due to cross-reaction of the C219 mAb with low levels of endogenous murine Pgp. RNA and protein expression correspond to each other for each of the clones analyzed (Fig 2, A and B). Thus, transcription variability is the main factor influencing Pgp and β-gal expression in pHaSMCZ-transduced cells.

Coordinate expression of Pgp and β-gal in pHaSMCZ-transduced cells

In previous studies,[6,7] we demonstrated that drug selection influences the expression of a bicistronic unit con-

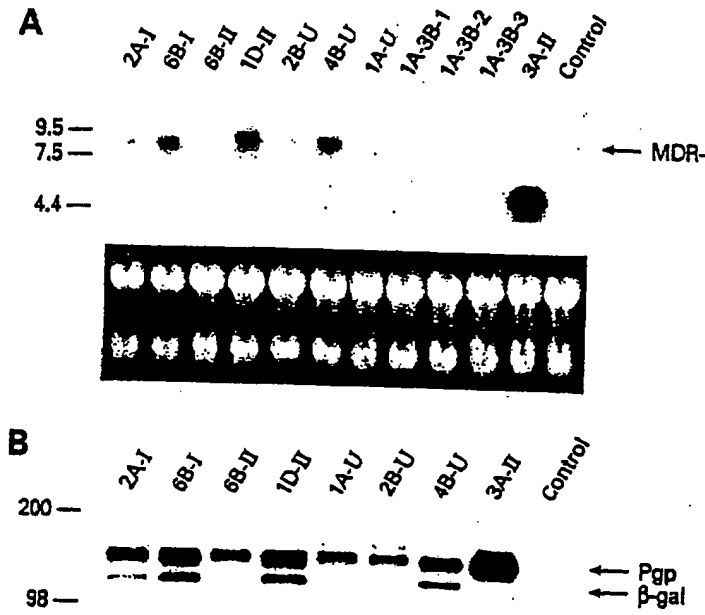

Figure 2. Coexpression of Pgp and β-gal in pHaSMCZ-transduced clones. NIH3T3 cells transduced with the pHaSMCZ retroviral vector were cloned by limiting dilution, and its expression was assessed by Northern blot analysis using an MDR-specific probe (A) and by 4–12% gradient sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotting using a mixture of Pgp-specific (C219) and β-gal-specific mAbs (B). The lower panel in A represents the ethidium bromide-stained gel prior to transfer. Numbers on the left correspond to the size of RNA molecular weight markers in kb (A) and of protein standards in kDa (B).

taining the *MDR1* selectable gene. To determine whether retroviral transfer of the long MDR-IRES-*lacZ* bicistronic transcript would impair drug-selected coexpression of Pgp and β-gal, we selected several of the transduced clones with different concentrations of drug. Figure 3 shows the result obtained for one representative clone (4B-U). Thus, coordinate expression of both Pgp and β-gal was maintained even at a high stringency of selection. Depending on the mRNA expression level for each clone, we could achieve a higher or lower stringency of drug selection, but in all cases increases in drug concentration correlated with enhancement in both Pgp and β-gal expression.

Long-term stability of the integrated MDR-IRES-lacZ proviruses in transduced NIH3T3 cells

To assess the stability of expression of the MDR-IRES-*lacZ* transductants in the absence of drug selection, one of the isolated single-cell clones (1A-U) was recloned twice by limiting dilution, and three of the third-generation single-cell clones, named 1A-3B-1, 1A-3B-2, and 1A-3B-3, were reanalyzed. By Southern blot analysis, a single band of 1.5 kb was present in *Nsi*I-restricted genomic DNA from the three subclones as well as the original 1A-U clone, using the HaM (B/N) probe[7] (data not shown). This observation confirmed that all subclones were derived from the original 1A-U clone. By Northern blot analysis (Fig 2A), only one of the three subclones (1A-3B-1) had expression levels of the bicistronic transcript comparable with the original 1A-U clone. Thus, at least for some clones, there is evidence of clonal instability in the expression of the bicistronic MDR-IRES-*lacZ* transcript over time.

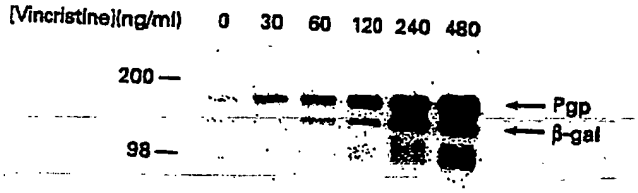

Figure 3. Drug selection increases the coordinate expression of Pgp and β-gal in pHaSMCZ-transduced cells. Cell extracts (20 μg protein/sample) from the single-cell clone 4B, transduced with the MDR-IRES-*lacZ* retrovirus, and selected at the indicated concentrations of vincristine, were fractionated by 8–16% gradient sodium dodecyl sulfate polyacrylamide gel electrophoresis and analyzed by immunoblotting with a mixture of anti-β-gal and C219 mAbs.

To assess whether this instability of expression could be overcome by drug selection of the unstable clonal populations, three different pHaSMCZ-transduced single-cell clones (1A-U, 2B-U, and 4B-U, see Fig 2) were chosen by virtue of their X-gal positive staining pattern. Although all three clones also expressed Pgp (see Fig 2), they had been selected solely on the basis of β-gal expression and had never been placed under cytotoxic pressure. Thus, 1A-U, 2B-U, and 4B-U cultures were evenly divided, and both resulting cultures were passaged in parallel over an 18-week period. To analyze the potential influence of the *MDR1* gene in maintaining sustained levels of expression of the β-gal passenger gene, one of the aliquots was challenged every 6th passage with 30 ng/mL vincristine for 4 days. Cells from each aliquot were harvested periodically just before drug selection and the β-gal expression of each aliquot was assessed by a specific ELISA that only detects the *E. coli* enzyme (see *Materials and Methods*). Figure 4 shows that for all three clones analyzed in the absence of cytotoxic selection there is a slow but progressive decrease in β-gal expression over time. Moreover, the kinetics of reduc-

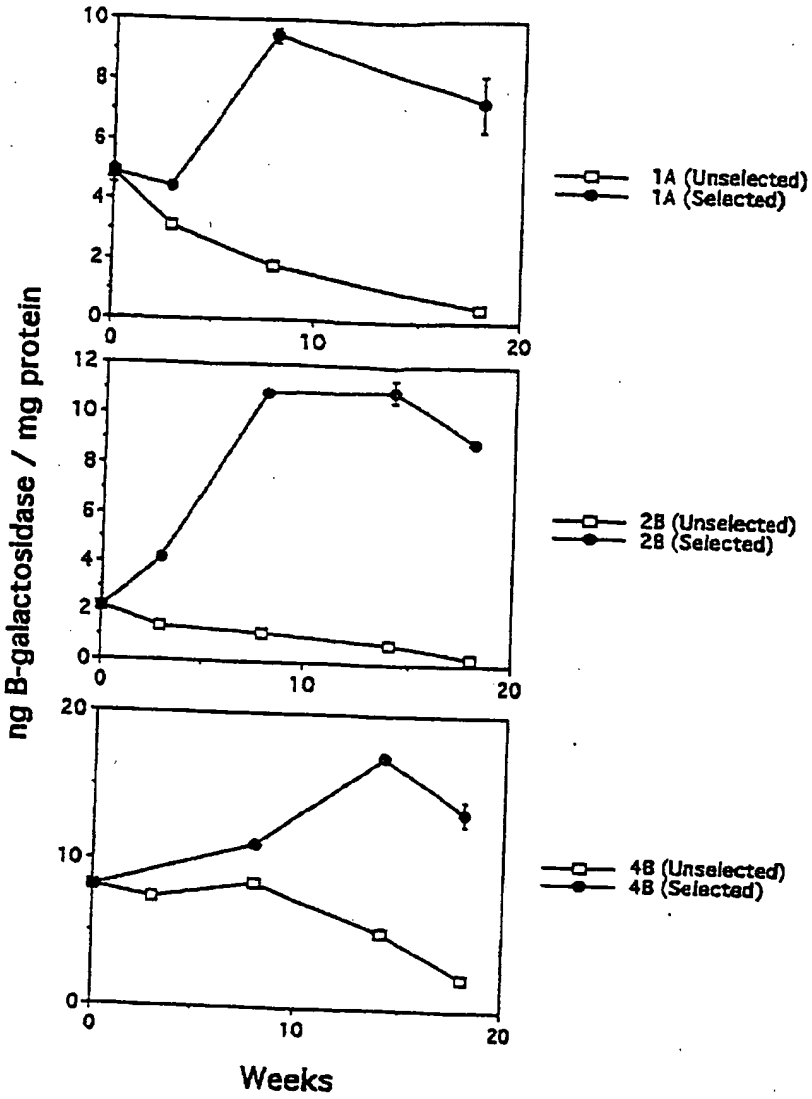

Figure 4. Periodic drug selection allows sustained β-gal expression on pHaSMCZ-transduced cells. Single-cell clones 1A, 2B, and 4B, transduced with the pHaSMCZ retrovirus, were split into two portions and passaged over an 18-week period without further manipulation (Unselected), or selected at week 3, 8, 14, and 18 with 30 ng/mL vincristine over 4 days (Selected). On the day prior to selection, both selected and unselected cells were collected and extracted. Finally, cell extracts were analyzed for *E. coli* β-gal expression by ELISA. Values are means ± SD of duplicate samples from two separate assays.

tion of β-gal expression is dependent upon the clone analyzed. Thus, clone 1A had a 91% reduction in β-gal expression in 18 weeks, and clone 2B had a 89% reduction, whereas the high-expressing clone 4B experienced a 74% decrease in β-gal expression. However, when the same clones were challenged periodically with a moderate dose of vincristine, their β-gal expression was not only sustained but could also be increased 2–5 times depending upon the clone analyzed.

In a similar experiment, cells from the three unselected clones (1A-U, 2B-U, and 4B-U) passaged for a 20-week period were finally challenged with 30 ng/mL vincristine for 6 days. For each clone, immunoblot analysis was used to compare levels of Pgp and β-gal expression before passaging (O), after prolonged passage (P), and after selection (S). Figure 5 shows that prolonged passage of cells that have integrated the MDR-IRES-*lacZ* provirus results in reduction of both Pgp and β-gal expression. This reduction of expression over time is clone-dependent and could be related to the site of chromosomal integration of the provirus. However, this progressive loss of expression can be fully reversed with only one round of cytotoxic selection in cells containing the *MDR1* gene. Thus, when the selectable *MDR1* gene is linked to a nonselectable gene such as β-gal, drug selection not only increases the levels of Pgp expression but also positively affects the expression levels of the passenger gene. For all three clones analyzed, there is barely detectable β-gal after prolonged passage of the unselected transductants (lanes P) which can be boosted by drug selection (lanes S) to levels equal or higher than those present in the original transductant clones (lanes O). The same result was obtained when original (O), passaged (P), and further selected (S) cells from each clone were analyzed for Pgp expression alone by fluorescence-activated cell sorter (FACS) analysis using the human Pgp-specific MRK-16 mAb[26] (data not shown).

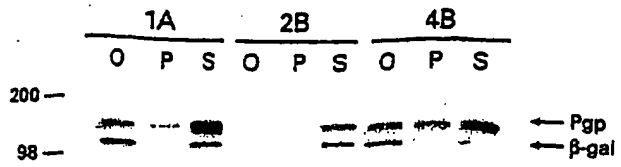

Figure 5. Modulation of Pgp and β-gal expression by drug selection in pHaSMCZ transductants. Samples from single-cell cloned, unselected 1A, 2B, and 4B transductants were obtained initially after isolation (O), after prolonged passage over a 20-week period (P), and from a 5-day selection with 30 ng/mL vincristine, subsequent to the 20-week passage (S). Cells were disrupted, electrophoresed in a 8–16% gradient gel (20 μg protein/lane), and analyzed by Western blotting with a mixture of anti-β-gal and anti-Pgp (C219) mAbs.

A second generation MDR-marking expression vector

We have found some limitations when using β-gal as reporter for the MDR phenotype (see *Discussion*). To overcome these limitations, we constructed a retroviral vector similar to pHaSMCZ, named pHaMCGFP, in which the Harvey murine sarcoma virus promoter drives the expression of a bicistronic transcript composed of the *MDR1* gene and the GFP as a reporter gene (Fig 1B). Translation of GFP is accomplished by the same IRES element used for β-gal expression from the pHaSMCZ vector. Thus, the reduced size of the MDR-IRES-GFP cassette (5.2 kb compared with 8 kb for the MDR-IRES-*lacZ* cassette) might increase the number of high titer pHaMCGFP producer clones.

To analyze function of the new pHaMCGFP retroviral vector, we stably transfected it into PA317 amphotropic packaging cells. Further selection of the transfected cells with 30 ng/mL vincristine yielded similar numbers of drug-resistant colonies for both pHaMCGFP and pHaMDR1/A, the control vector, at a frequency of $\sim 1.0 \times 10^{-3}$. To assess whether these transfected, drug-selected cells expressed a functional GFP in sufficient amounts to be easily detected, we examined the cultured cells by fluorescence microscopy. Non-transfected PA317 cells and pHaMDR1/A controls did not show any fluorescence (data not shown). In contrast, virtually all pHaMCGFP-transfected PA317 cells that were further selected at 30 ng/mL vincristine exhibited enough GFP expression to be easily visualized by fluorescence microscopy with a standard fluorescein isothiocyanate filter. Again, similar to the X-gal staining pattern obtained for β-gal-expressing cells transfected with pHaSMCZ, a heterogeneous pattern of fluorescence was seen (see Fig 6). Moreover, when these same cells were detached from the dishes by trypsin and visualized in suspension, their fluorescence intensity became brighter than when the cells were attached to the dishes.

Next, we determined whether FACS analysis could also be used to distinguish the pHaMCGFP-transfected PA317 cells from the untransfected PA317 cells. The histogram of Figure 7A shows that indeed pHaMCGFP-transfected PA317 cells selected with 30 ng/mL vincristine were >1 log brighter than their untransfected

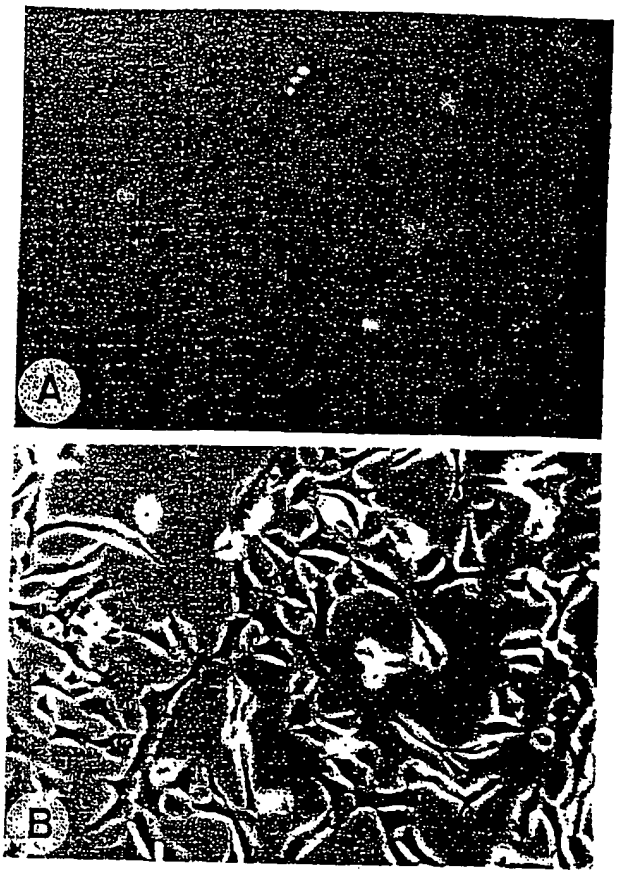

Figure 6. GFP detection in pHaMCGFP transfectants. PA317 cells were transfected with 10 μg of pHaMCGFP by calcium phosphate coprecipitation and selected with 30 ng/mL vincristine at 48 hours post-transfection. A: drug-resistant, stable pHaMCGFP transfectants visualized (×40 magnification) using the fluorescein isothiocyanate filter set (450–490 nm). B: same as A with additional phase contrast lighting.

counterparts. To demonstrate that the expression of GFP was related to that of the *MDR1* gene product Pgp, the pHaMCGFP-transfected cells were selected at increasing drug concentrations and the corresponding drug-resistant colonies were pooled and analyzed either by FACS (Fig 7A) or by Western blotting with anti-Pgp C219 mAb and anti-GFP antiserum (Clontech, Palo Alto, Calif) (data not shown). Both experiments demonstrated coordinate expression of Pgp and GFP from the MDR-IRES-GFP bicistronic cassette.

Finally, to assess whether these cells had become producers capable of synthesizing retroviral particles carrying the bicistronic pHaMCGFP retrovirus, NIH3T3 cells were infected with supernatant from the pooled producers. Figure 7B indicates that recombinant virus particles from the pHaMCGFP producer pool were capable of transducing target fibroblasts and transferred both the MDR phenotype and GFP-related fluorescence to these cells. We estimated a titer of 5×10^4 cfu/mL by drug selection when analyzing the supernatant from

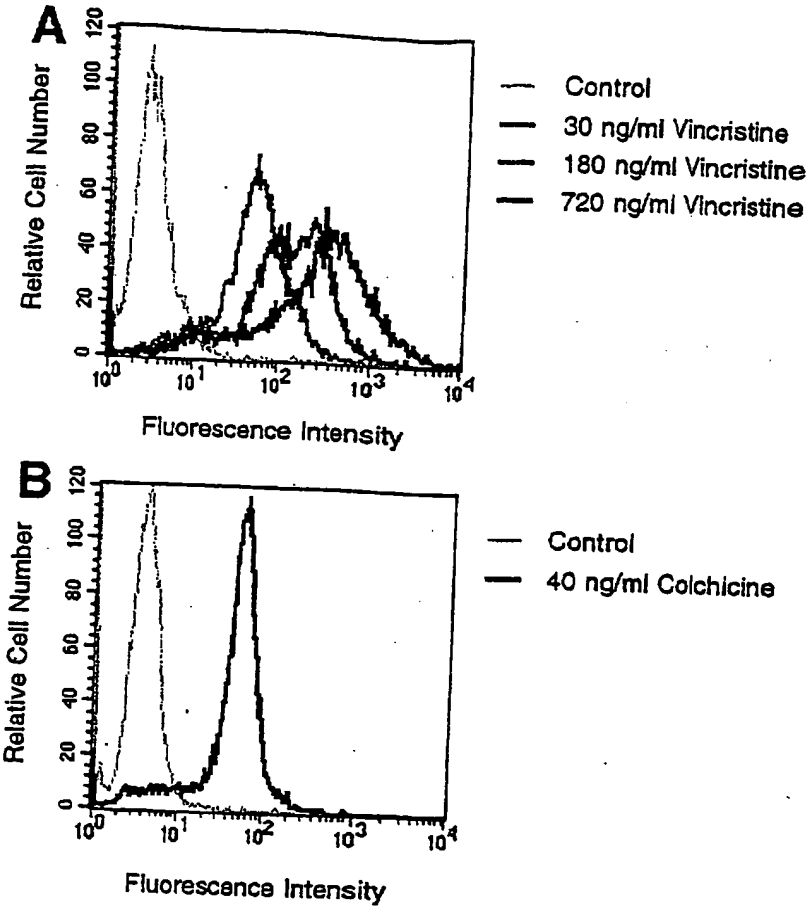

Figure 7. FACS analysis of GFP expression in pHaMCGFP cells. GFP expression correlates with drug resistance in pHaMCGFP transfectants (A). Amphotropic PA317 packaging cells were transfected with pHaMCGFP, selected at the indicated concentrations of vincristine, and analyzed in a FACSort as described in *Materials and Methods*. The FL1 emission channel was used to monitor green fluorescence. The result is presented as histograms overlaid on a control histogram from PA317 cells transfected with the control plasmid pHaMDR1/A and selected with 720 ng/mL vincristine. B: GFP expression in drug- selected pHaMCGFP transductants. Supernatants from pHaMCGFP-transfected PA317 cells were used to transduce NIH3T3 cells, which were subsequently selected with 40 ng/mL colchicine. The drug-selected sample histogram is overlaid on a control histogram from untransduced NIH3T3 cells.

pooled, pHaMCGFP-transfected, PA317 amphotropic producers.

DISCUSSION

The rationale behind the use of the *MDR*1 gene for gene transfer is to allow the selection of drug-sensitive tissues such as bone marrow, which have received the *MDR*1 gene, with a wide spectrum of clinically relevant cytotoxic drugs such as Taxol, doxorubicin, and vinblastine. A possible direct therapeutic benefit of *MDR*1 gene expression would be protection against aggressive chemotherapy regimens in cancer patients. Moreover, when linked to a therapeutic gene, its ability to confer resistance to cytotoxic drugs makes the *MDR*1 gene a promising candidate as a dominant selectable gene for gene therapy.

Although multiple *in vitro* studies have defined the role of the *MDR*1 gene in the MDR phenotype, no reports to date have evaluated its efficacy as a transgene for coselection of gene expression *in vivo*. This paper reports the construction and *in vitro* characterization of two different MDR reporting systems that should facilitate the study of *MDR*1-selectable gene transfer and expression *in vivo*.

E. coli β-gal as a reporter of MDR1 gene expression: vector design

When using a selectable gene such as the *MDR*1 gene in retroviral vectors for increased proviral stability, our bicistronic system is more desirable than bicistronic vectors with two separate promoters as discussed previously.[6] In this study, we have transcriptionally linked the *E. coli lacZ* gene to the human *MDR*1 gene and placed this bicistronic cassette under the control of the strong Harvey murine sarcoma virus retroviral promoter (HaLTR). As a genetic reporter molecule, β-gal is frequently used, and it is still one of the most versatile of these molecules due to the rapid, easy, and sensitive detection of its enzymatic activity.[27] Colorimetric, fluorometric, and chemiluminescent assays are available. However, one important limitation of the *MDR1/lacZ* gene in a bicistronic unit is the considerable size of this gene fusion (8 kb), which relates to the insert capacity of retroviral vectors.[22,23] We anticipated that packaging and/or expression of the MDR-IRES-*lacZ* transcript

would be impaired, at least in the original pHaL retroviral backbone[15] which already contained 725 nucleotides of rat endogenous VL30 sequences flanking the insert position upstream and nearly 3000 nucleotides of these sequences downstream.[28] Therefore, we analyzed two new versions of the Harvey murine sarcoma retroviral backbone with different deletions in the VL30 sequences.

Although both deleted versions as well as the original backbone were capable of directing expression of the MDR-IRES-*lacZ* cassette with similar efficiency, only the shorter backbone, pHaS,[16] was able to produce functional virus particles. This indicates that packaging of the MDR-IRES-*lacZ* bicistronic transcript requires elimination of the rat VL30 sequences downstream from the insert. Conversely, the remaining upstream VL30 sequences contain *cis*-acting packaging and dimerization domains; RNAs including these sequences are necessary and either appear to be packaged more efficiently or are more stable than RNAs with analogous Moloney murine leukemia virus sequences.[29] Velu et al[30] reported evidence of a 400-bp fragment with enhancer activity based within the 1-kb portion of rat VL30 sequences that we removed in pHaM. Consistent with the result obtained by Metz et al,[16] we have not obtained evidence of such a putative enhancer because the transfection efficiencies of pHaLMCZ, pHaMMCZ, and pHaSMCZ were quite similar.

After intensive screening for high-titer ecotropic and amphotropic producer clones, we could only obtain single-cell producer clones with modest titers of ~0.5–1.0 × 10^4 cfu/mL. This may reflect inefficient packaging or reduced stability of the long MDR-IRES-*lacZ* bicistronic transcriptional fusion. It should be noted that in the few isolated single-cell producer clones synthesizing virus particles with deleted versions of the *lacZ* gene, their titers and expression levels were at least 10-fold higher than those synthesizing virus particles containing the complete MDR-IRES-*lacZ* cassette (data not shown). Metz et al[16] have also reported a 5- to 10-fold reduction in titers when comparing the original pHaL and the reduced pHaS backbones for both transfer and expression of the *MDR*1 gene. However, in an analogous experiment, we have not obtained any drop in titers when comparing the pHaM backbone with the original pHaL backbone (data not shown). These data suggest that downstream VL30 sequences, but not those deleted in the pHaM backbone, may contribute to efficient virus production.

Factors affecting stability of β-gal reporter gene expression

Since many gene therapy studies seek long-term complementation of genetic defects in target cells, it is important in any model system to understand the factors that may influence provirus stability and expression in addition to vector design. Such factors include the nature of the integration site, the nature of the reporter and selectable genes, the presence or absence of selection pressure, and the nature of the target cell.

Under the low multiplicity of infection conditions used in this study, most transduced cells would be expected to contain a single copy of the integrated provirus. Thus, variability of expression among the clones studied was shown to be at the transcriptional level only, because similar variability was seen when analyzing the relative amounts of Pgp and β-gal synthesized from the same clones (Fig 2). Transcriptional variability in retroviral vectors has been shown to be dependent upon the integration site.[31,32] Thus, it is conceivable that for long transcripts, such as that synthesized from our MDR-IRES-*lacZ* bicistronic cassette, not only the strength of the promoter but also the stability of the integrated provirus could be strongly influenced by the chromosomal integration site.

At the single-cell level, heterogeneity in the pattern of X-gal staining was present within different cells derived from a single-cell clone, whether selected or not. Since selected cells should all express Pgp, this intraclonal heterogeneity might reflect an epigenetic mechanism involving the IRES element, which directly affects β-gal expression. Despite this heterogeneity of β-gal expression within clones, increased coordinate expression of both Pgp and β-gal was observed in clonally derived populations by increasing the selective pressure in all clones analyzed, similar to previously reported results.[6,7] Thus, selection of populations should lead to an "average" increase in expression of a passenger gene.

In this study we document a slow but progressive loss of both Pgp and β-gal expression over time in cells that otherwise retain the integrated MDR-IRES-*lacZ* proviruses. Thus, this kind of instability is not related to any chromosomal loss. Moreover, this provirus shutdown, whether position-dependent or influenced by epigenetic gene regulatory processes such as methylation, is circumvented by drug selection with Pgp substrates. Therefore, in the MDR-based bicistronic system, periodic pulses of selective pressure are helpful in maintaining provirus structural and functional integrity.

Upon transduction of pHaSMCZ into NIH3T3 cells with the C-6 amphotropic producer line, we have found expression of the full-length bicistronic MDR-IRES-*lacZ* transcript in the majority of clones analyzed (Fig 2A). Also, we found no evidence for the utilization of cryptic splice sites in the *MDR*1 gene, contrary to data reported by Sorrentino et al[33] in hematopoietic cells from mice. This difference may reflect cell type-specific differences in *MDR*1 mRNA splicing.

GFP as a reporter of MDR1 gene expression

By linking both Pgp and GFP expression in a bicistronic retrovirus, we have achieved efficient and stable marking of the MDR phenotype. GFP is a 27-kDa polypeptide that generates a very stable fluorescent chromophore by oxidation. This fluorescence is species-independent and does not require cofactors, substrates, or additional gene

products to allow detection in living cells. Cheng et al[34] and Levy et al[35] have recently shown efficient expression of a humanized, red-shifted GFP when introduced through retroviral vectors into different cell types. Our vector, similar to their vectors, included an improved version of GFP. The two new features of this second-generation GFP are a S65T gain of function mutant, which results in a red-shifted excitation peak with increased brightness, and the conversion of jellyfish GFP codons to human codon usage, which results in higher expression levels because of a more efficient translation in mammalian cells. Using the humanized, red-shifted GFP, a single copy of the integrated viral genome has been shown to be sufficient for production of detectable fluorescence.

We previously synthesized a retroviral vector identical with the MDR-IRES-GFP vector by using the wild-type GFP (wtGFP) but were unable to detect any fluorescence either by FACS analysis or microscopy, even when the transfected or transduced cells were stringently selected with high concentrations of drug (data not shown). wtGFP has been shown to display much less green fluorescence than the optimized, humanized, red-shifted GFP[34,35], and therefore the level of wtGFP expression obtained from our MDR-IRES-wtGFP vector was not enough to produce a detectable signal. We have also found that GFP expression from our retroviral system is nontoxic to the cells, as has been previously reported.[34,35]

By linking the starting codon of GFP to the 3′-end of the IRES sequence through an *Nco*I site, we changed the amino acid next to the initiator Met from Ser to Gly. From the recently solved crystal structure of recombinant GFP,[36] it has been determined that small changes or extensions at either the N terminus or the C terminus would not disrupt the structure of the protein. Furthermore, Clontech has incorporated this same mutation in some of its commercial GFP-based plasmids (Living Colors™, GFP Application Notes booklet).

The optimized GFP gene has several advantages over other reporter genes, such as the *lacZ* gene. Intrinsic limitations of the latter include: (a) the endogenous β-gal activities in some mammalian cells. We have tried to detect pHaSMCZ-transduced mouse fibroblasts *in vivo* using the fluorogenic β-gal substrate fluorodeoxyglucose but have been unsuccessful because of the high fluorescent background produced in these cells. (b) The large size of the *lacZ* gene, which, as previously shown, leads to reduced titers on MDR-based retroviral vectors. The significantly smaller size of the GFP gene facilitates the construction of high-titer MDR-IRES-GFP producer clones. We have obtained titers of 5×10^4 cfu/mL from supernatants of the original pHaMCGFP-transfected PA317 amphotropic pool. Based on the results obtained using a similar vector and the same PA317 packaging cell line,[7] screening for high-titer pHaMCGFP producer clones from the initial transfected pool should yield at least a 10-fold increase in the final titers of producer clones synthesizing the pHaMCGFP retrovirus.

In addition, the use of GFP is extremely convenient and allows rapid marking of the MDR phenotype over classical detection methods such as immunological detection of Pgp with the MRK-16 mAb, which are considered to be invasive, time-consuming, and/or more nonspecific. Further improvements in the sensitivity of detection of GFP will widen the use of this outstanding reporter gene for the most demanding biological applications.

Experimental and clinical applications of pHaSMCZ and pHaMCGFP retroviral vectors

A potential advantage of MDR-based vectors for gene therapy is that *in vivo* selection could allow survival and expansion of even rare transduction events. Thus, the MDR-IRES-*lacZ* and the MDR-IRES-GFP transcriptional fusions, when introduced into a retrovirus or another efficient eukaryotic expression vector, may become excellent systems to study *in vivo* gene transfer and expression of the *MDR*1 gene in human clinical trials.

The endogenous *MDR*1 gene is expressed in primitive hematopoietic cells at low levels, and its expression is extinguished with myeloid maturation.[37] Therefore, the *MDR*1-reporter gene fusions that we have synthesized should be useful to monitor both directly and at the single-cell level the extent of *MDR*1 gene transfer and its short- and long-term expression into hematopoietic stem cells *in vivo*, the effect of transgenic *MDR*1 expression on the stem cell phenotype (e.g., engraftment capability), and the spread of the acquired MDR phenotype through the different hematopoietic lineages. The linked reporters would also facilitate distinguishing the transgenic from the endogenous MDR phenotype. Moreover, fast and noninvasive reporter gene tracking procedures would complement other analyses (e.g., reverse transcriptase PCR, immunofluorescence, rhodamine efflux) that have been successfully employed to estimate some of the parameters mentioned.[38]

Another application of the MDR-IRES-*lacZ* and MDR-IRES-GFP chimeras is to optimize chemotherapy regimens (including new cytotoxic Pgp substrates and reversing agents) used to select *MDR*1-transduced cells *in vivo*. The association of these reporter genes with an acquired MDR phenotype should help in evaluating the potential of the *MDR*1 gene to attenuate the myelosuppression associated with chemotherapy and to enrich and expand genetically modified blood cells by dominant selection. Several clinical trials have recently been initiated to determine to what extent drug-resistant hematopoiesis can be achieved in adult patients with metastatic breast or ovarian cancer. In addition, preliminary results from these clinical trials have provided insight into relevant cell biology questions related to hematopoiesis and stem cell biology, such as the fact the granulocyte-macrophage CFU cells do not contribute to post-transplant hematopoietic recovery following intensive systemic chemotherapy.[39] Thus, these clinical trials might also provide benefits by incorporating our MDR-

marking vectors to help track the presence of the *MDR1* gene in hematopoietic cells and other cell types.

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REFERENCES

1. Gottesman MM, Pastan I. Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu Rev Biochem.* 1993;62:385–427.
2. Gottesman MM, Hrycyna, CA, Pastan I. Genetic analysis of the multidrug transporter. *Annu Rev Genet.* 1995;29: 607–649.
3. Ford JM, Hait WN. Pharmacology of drugs that alter multidrug resistance in cancer. *Pharmacol Rev.* 1990;42: 155–199.
4. Ford JM, Hait WN. Pharmacologic circumvention of multidrug resistance. *Cytotechnology.* 1993;12:171–212.
5. Gottesman MM, Pastan I. The multidrug resistance (*MDR1*) gene as a selectable marker in gene therapy. In: Cohen-Haguenauer O., Boiron M., eds. *Human Gene Transfer.* Paris: Colloque INSERM/John Libbey Eurotext Ltd. 1991;219:185–191.
6. Aran JM, Gottesman MM, Pastan I. Drug-selected coexpression of human glucocerebrosidase and P-glycoprotein using a bicistronic vector. *Proc Natl Acad Sci USA.* 1994; 91:3176–3180.
7. Aran JM, Licht T, Gottesman MM, Pastan I. Complete restoration of glucocerebrosidase deficiency in Gaucher fibroblasts using a bicistronic MDR retrovirus and a new selection strategy. *Hum Gene Ther.* 1996;7:2165–2175.
8. Sugimoto Y, Aksentijevich I, Murray GJ, et al. Retroviral coexpression of a multidrug resistance gene (*MDR 1*) and human α-galactosidase A for gene therapy of Fabry disease. *Hum Gene Ther.* 1995;6:905–915.
9. Galski H, Sullivan M, Willingham MC, et al. Expression of a human multidrug resistance cDNA (MDR 1) in the bone marrow of transgenic mice: resistance to daunomycin-induced leukopenia. *Mol Cell Biol.* 1989;9:4357–4363.
10. Mickisch GH, Merlino GT, Galski H, et al. Transgenic mice that express the human multidrug-resistance gene in bone marrow enable a rapid identification of agents that reverse drug resistance. *Proc Natl Acad Sci USA.* 1991;88: 547–551.
11. Sorrentino BP, Brandt SJ, Bodine D, et al. Selection of drug-resistant bone marrow cells in vivo after retroviral transfer of human MDR1. *Science.* 1992;257:99–103.
12. Podda S, Ward M, Himelstein A, et al. Transfer and expression of the human multiple drug resistance gene into live mice. *Proc Natl Acad Sci USA.* 1992;89:9676–9680.
13. Hanania EG, Deisseroth AB. Serial transplantation shows that early hematopoietic precursor cells are transduced by MDR1 retroviral vector in a mouse gene therapy model. *Cancer Gene Ther.* 1994;1:21–25.
14. Zolotukhin S, Potter M, Hauswirth WW, et al. A "humanized" green fluorescent protein cDNA adapted for high level expression in mammalian cells. *J Virol.* 1996;70:4646–4654.
15. Pastan I, Gottesman MM, Ueda K, Lovelace E, Rutherford AV, Willingham MC. A retrovirus carrying an *MDR1* cDNA confers multidrug resistance and polarized expression of P-glycoprotein in MDCK cells. *Proc Natl Acad Sci USA.* 1988;85:4486–4490.
16. Metz MZ, Best DM, Kane SE. Harvey murine sarcoma virus/*MDR1* retroviral vectors: efficient virus production and foreign gene transduction using MDR1 as a selectable marker. *Virology.* 1995:208:634–643.
17. Miller AD, Buttimore C. Redesign of retrovirus packaging cell lines to avoid recombination leading to helper virus production. *Mol Cell Biol.* 1986;6:2895–2902.
18. Markowitz D, Goff S, Bank A. A safe packaging line for gene transfer: separating viral genes on two different plasmids. *J Virol.* 1988;62:1120–1124.
19. Danos O, Mulligan RC. Safe and efficient generation of recombinant retroviruses with amphotropic and ecotropic host ranges. *Proc Natl Acad Sci USA.* 1988;85: 6460–6464.
20. Aran JM, Germann UA, Gottesman MM, Pastan I. Construction and characterization of a selectable multidrug resistance-glucocerebrosidase fusion gene. *Cytokines Mol Ther.* 1996;2:47–57.
21. Kartner N, Evernden-Porelle D, Bradley G, Ling V. Detection of P-glycoprotein in multidrug resistant cell lines by monoclonal antibodies. *Nature.* 1985;316:820–823.
22. Friedmann T. Progress toward human gene therapy. *Science.* 1989;244:1275–1281.
23. Jolly D. Viral vector systems for gene therapy. *Cancer Gene Ther.* 1994;1:51–64.
24. Hoeben RC, Migchielsen AA, van der Jagt RC, et al. Inactivation of the Moloney murine leukemia virus long terminal repeat in murine fibroblast cell lines is associated with methylation and dependent on its chromosomal position. *J Virol.* 1991;65:904–912.
25. Kim DG, Kang HM, Jang SK, Shin HS. Construction of a bifunctional mRNA in the mouse by using the internal ribosomal entry site of the encephalomyocarditis virus. *Mol Cell Biol.* 1992;12:3636–3643.
26. Hamada H, Tsuruo T. Functional role for the 170- to 180-kDa glycoprotein specific to drug-resistant tumor cells as revealed by monoclonal antibodies. *Proc Natl Acad Sci USA.*. 1986;83:7785–7789.
27. Alam J, Cook JL. Reporter genes: application to the study of mammalian gene transcription. *Anal Biochem.* 1990; 188:245–254.
28. Ellis RW, DeFeo D, Maryak JM, et al. Dual evolutionary origin for the rat genetic sequences of Harvey murine sarcoma virus. *J Virol.* 1980;36:408–420.
29. Torrent C, Gabus C, Darlix J-L. A small and efficient dimerization/packaging signal of rat VL30 RNA and its use in murine leukemia virus VL30-derived vectors for gene transfer. *J Virol.* 1994;68:661–667.
30. Velu TJ, Vass WC, Lowy DR, Tambourin PE. Harvey murine sarcoma virus: influences of coding and noncoding sequences on cell transformation *in vitro* and oncogenicity *in vivo*. *J Virol.* 1989;63:1384–1392.
31. Davies RL, Fuhrer-Krusi S, Kucherlapati R. Modulation of transfected gene expression mediated by changes in chromatin structure. *Cell.* 1982;31:521–529.
32. Freinstein SC, Ross SR, Yamamoto K. Chromosome position effects determine transcriptional potential of integrated mammary tumor virus DNA. *J Mol Biol.* 1982; 156:549–565.
33. Sorrentino BP, McDonagh KT, Woods D, Orlic D. Ex-

pression of retroviral vectors containing the human *MDR1* cDNA in hematopoietic cells of transplanted mice. *Blood.* 1995;86:491–501.

34. Cheng L, Fu J, Tsukamoto A, Hawley RG. Use of green fluorescent protein variants to monitor gene transfer and expression in mammalian cells. *Nat Biotechnol.* 1996;14: 606–609.
35. Levy JP, Muldoon RR, Zolotukhin RR, Link CJ, Jr. Retroviral transfer and expression of a humanized, red-shifted green fluorescent protein gene into human tumor cells. *Nat Biotechnol.* 1996;14:610–614.
36. Yang F, Moss LG, Phillips GN, Jr. The molecular structure of green fluorescent protein. *Nat Biotechnol.* 1996;14: 1246–1251.
37. Chaudhary PM, Roninson IB. Expression and activity of P-glycoprotein, a multidrug efflux pump, in human hematopoietic stem cells. *Cell.* 1991;66:85–94.
38. Hanania EG, Fu S, Zu S, et al. Chemotherapy resistance to Taxol in clonogenic progenitor cells following transduction of CD34 selected marrow and peripheral blood cells with a retrovirus that contains the MDR-1 chemotherapy resistance gene. *Gene Ther.* 1995;2:285–294.
39. Hanania EG, Giles RE, Kavanagh J, et al. Results of MDR-1 vector modification trial indicate that granulocyte/macrophage colony-forming unit cells do not contribute to posttransplant hematopoietic recovery following intensive systemic therapy. *Proc Natl Acad Sci USA.* 1996;93:15346–15351.

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Green Fluorescent Protein Retroviral Vectors: Low Titer and High Recombination Frequency Suggest a Selective Disadvantage

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ABSTRACT

Green fluorescent protein (GFP) has been used as a reporter molecule for gene expression because it fluoresces green after blue-light excitation. Inclusion of this gene in a vector could allow rapid, nontoxic selection of successfully transduced cells. However, many attempts by our laboratory to isolate stable retroviral producer cell clones secreting biologically active vectors containing either the highly fluorescent S65T-GFP mutant or humanized GFP have failed. Vector plasmids containing various forms of GFP and the neomycin resistance gene were transfected into three different packaging cell lines and fluorescence was observed for several days, but stable clones selected with G418 no longer fluoresced. Using confocal microscopy, the brightest cells were observed to contract and die within a matter of days. RNA slot-blot analysis of retroviral producer supernatants showed no viral production from the GFP plasmid-transfected clones, although all clones derived after transfection with an identical retroviral construct not containing GFP produced virus. Genomic Southern analysis of the GFP-transduced clones showed a much higher probability of rearrangement of the priviral sequences than in the control non-GFP clones. Overall, 18/34 S65T-GFP clones and 17/33 humanized-GFP clones had rearrangements, whereas 2/15 control non-GFP clones had rearrangements. Hence, producer cells expressing high levels of these GFP genes seem to be selected against, with stable clones undergoing major rearrangements or other mutations that both abrogate GFP expression and prevent vector production. These observations indicate that GFP may not be an appropriate reporter gene for gene transfer applications in our vector/packaging system.

OVERVIEW SUMMARY

In this study, we systemically examined the production of retroviral vectors expressing green fluorescent protein (GFP) and report that, despite the production of low levels of vector from bulk populations of fluorescing packaging cells soon after transfection, stable clones produced neither virus nor GFP. A significantly higher frequency of rearrangement of the proviral sequences in the stable clones was found by genomic Southern analysis compared to control producer cell lines. This implies a selective advantage for those clones that rearrange the GFP gene and abrogate its expression. Hence, GFP may not be a suitable selective marker for mammalian gene transfer applications in our vector/packaging system.

INTRODUCTION

GREEN FLUORESCENT PROTEIN (GFP) is derived from the jellyfish *Aequoria victoria*. The GFP cDNA encodes a 238-amino-acid protein with a molecular weight of 27 kD (Prasher *et al.*, 1992). Blue light stimulates the excitation and green fluorescence from a cyclized GFP fluorophore formed by serine-65, tyrosine-66, and glycine-67. Light-stimulated fluorescence does not require co-factors, substrates, or additional gene products, unlike other fluorescent markers. The signal is detectable by both fluorescent microscopy or fluorescence-activated cell sorting (FACS) analysis of live cells (Chalfie *et al.*, 1994). However, the fluorescence of wild-type GFP after visible spectrum excitation is not strong enough for most applications. Therefore, variants of GFP, such as S65T-GFP in which ser-

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ine-65 is altered to threonine, have been developed resulting in a red-shifted excitation peak and four- to six-fold improvement in emission intensity (Heim *et al.*, 1995). Recently, another mutant GFP, humanized S65T-GFP (hGFP), was derived to optimize human-type codon usage, possibly resulting in the improved translation of the gene (Levy *et al.*, 1996; Zolotukhin *et al.*, 1996).

In the field of gene transfer technology, marker genes allowing direct and simple detection of successfully transduced cells would be extremely useful (Kain and Ganguly, 1995). The possibility of immediately selecting for successfully transduced viable cells by simple FACS would be especially welcome in the field of gene transfer to hematopoietic stem and progenitor cells (Dunbar and Emmons, 1994). Gene transfer efficiencies, even with widely used and well-developed murine retroviral vectors, are very low and an ability to select and possibly expand transduced cells could greatly improve the possibility for real therapeutic applications. Detection of marker gene products such as β-galactosidase (β-Gal), luciferase, chloramphenicol acetyltransferase (CAT), or alkaline phosphatase involves either cell fixation, which kills the cells, or antibody-mediated detection, which is time-consuming and can be prone to high background. Drug resistance genes allow positive selection of transduced cells only with days to weeks of growth in selective media, likely changing the characteristics of the target cells through terminal differentiation or other processes. These problems have stimulated investigators to search for a better gene marker system that provides timely, accurate, and nontoxic detection of successful transduction in living cells. One of the candidates is GFP.

We have attempted to isolate stable retroviral producer cell lines packaging a retroviral vector containing the GFP gene and the neomycin resistance (*neo*) gene. In this report, we demonstrate that stable clones producing GFP vectors could not be isolated after drug selection despite initial detection of GFP expression in the packaging cell lines. The G418-resistant clones were found to have rearranged at high frequency, precluding GFP expression and vector production, presumably due to a strong selective advantage for loss of GFP expression.

MATERIALS AND METHODS

Plasmid construction

The plasmid pG1Na (Genetic Therapy Inc., Gaithersburg, MD) served as the backbone for our GFP vectors. For pG1NGFP, a splice donor site was inserted 5′ to the *neo* gene, and a splice acceptor site and the S65T variant of the GFP gene (Clontech, Palo Alto, CA) were introduced 3′ of the *neo* gene such that both genes were driven from the 5′ long terminal repeat (LTR). pG1XGFP was produced by inserting the S65T-GFP gene 5′ of the *neo* gene in pG1Na under the control of the 5′-LTR. An SV40 immediate early promoter was then inserted 5′ of the *neo* sequence to drive this gene (Fig. 1). pG1NhGFP was then constructed by replacing the S65T-GFP gene in pG1NGFP with the humanized form of the S65T-GFP (hGFP) gene (Clontech, Palo Alto, CA).

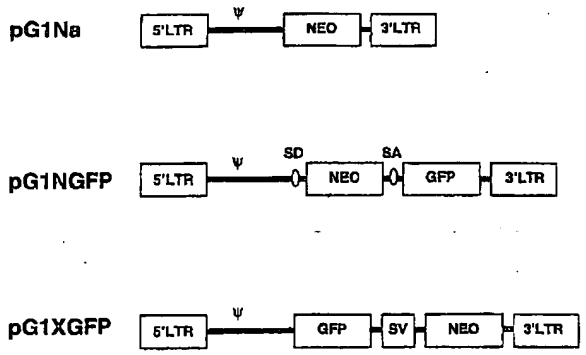

FIG. 1. Retroviral plasmid constructs. The backbone of the retroviral constructs was pG1Na. Two retroviral constructs, both expressing S65T-GFP from the 5′ LTR promoter, were produced. In one construct, a *neo* gene shared the 5′ LTR promoter through a splice donor-acceptor site (pG1NGFP) and in the other construct *neo* was driven by an SV40 promoter independently (pG1XGFP). LTR, long terminal repeat; GFP, S65T form of the GFP gene; NEO, neomycin resistance gene; SV, SV40 immediate early promoter; SD, splice donor site; SA, splice acceptor site; Ψ, viral packaging signal.

Retroviral producer cell lines

Ψ-CRIP (Danos and Mulligan, 1988) and PA317 (Miller and Buttimore, 1986) are murine amphotropic retroviral packaging cell lines. Ψ-2 (Mann *et al.*, 1983) is a murine ecotropic retroviral packaging cell line. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum (GIBCO-BRL, Gaithersburg, MD) at 37°C and 5% CO_2. Ψ-CRIP, PA317, and Ψ-2 cells were transfected with plasmids by the lipofectin method according to the manufacturer's protocol (GIBCO-BRL). After 48 hr, some plates were exposed to media containing 400 μg/ml G418 (active) (GIBCO-BRL) and other plates were split 1:5, 1:10, 1:20, 1:50, and 1:100 into the G418-containing selective media. The cells were grown for 10–14 days and either bulk G418-resistant or macroscopic individual clonal populations were expanded for further study. Clones were grown for 2–4 weeks before culture supernatant was harvested for slot-blot hybridization and DNA was extracted for Southern blot analysis.

G1Na.40 is a cloned amphotropic G1Na-producer cell line derived from PA317 (Genetic Therapy Inc., Gaithersburg, MD) with a titer of 1–5 × 10^6 particles/ml.

Shuttle packaging experiments

We used the BOSC23 cells for transient expression of retroviruses (Pear *et al.*, 1993). The cells were transfected with plasmids by the calcium phosphate method described elsewhere (Pear *et al.*, 1993). Two days after transfection, the supernatants containing viruses were collected and filtered. Virus production from the BOSC23 cells was confirmed by the slot-blot analysis, as described below. Packaging cells were infected with the supernatants of the BOSC23 cells as previously described (Migita *et al.*, 1995).

Fluorescent microscopy

A fluorescent confocal microscope with a mercury arc lamp (100 watt) and a fluorescent filter set (consisting of a 480/40-nm excitation filter and a 535/50-nm emission filter) was used to detect GFP in living cells (Nikon, Melville, NY).

Slot-blot analysis

Supernatants from 70–90% of confluent producer cells were filtered through 0.45-μm filters. One milliliter of each supernatant was precipitated with 30% polyethylene glycol 8000 (Fisher Scientific, Fair Lawn, NJ) and the pellets were resuspended with 200 μl of Tris-EDTA buffer (ph 7.4) containing 10% (wt/vol) vanadyl ribonuclease complex (Gibco BRL, Gaithersburg, MD) and 100 μg/ml of yeast tRNA (GIBCO-BRL) and then lysed by adding 200 μl of 2× lysis buffer (1% SDS, 0.6 *M* NaCl, 20 m*M* EDTA, and 20 m*M* Tris-HCl pH 7.4). Retroviral RNA was extracted from the lysed solution by phenol. The RNA was reconstituted with 500 μl of a 7.5% formaldehyde solution containing 1.5 *M* NaCl and 150 m*M* sodium citrate pH 7.0. Next, 100 μl and 400 μl of each RNA sample were loaded into adjacent wells of the slot-blot apparatus Minifold II (Schleicher & Schuell, Keene, NH) with vacuum applied. The transfer membrane (Hybond N^+, Amersham, Cleveland, OH) was hybridized with a *neo* probe or GFP probe generated by PCR. The *neo* gene primers are 5′-ATGATTGAACAAGATGGATTGCA-3′ and 5′-AGGCATCGCCATGGGTCACGACGAGAT-3′. The primer sequences for the S65T-GFP gene are 5′-CTGGA-GTTGTCCCAATTCTTGTTG-3′ and 5′-TCAAGAAGGAC-CATGTGGTCTCTC-3′, and those for the humanized S65T-GFP gene are 5′-TGAACGGCCACAAGTTCAGCGTGT-3′ and 5′-TTACTTG- TACAGCTCGTCCATGCC-3′. Radiolabeling of the probes was done using an oligolabeling kit (Pharmacia, Piscataway, NJ).

Southern blotting

Nuclear DNA was extracted from producer cells of NIH-3T3 cells using a nonorganic DNA extraction kit (Oncor, Gaithersburg, MD). Ten micrograms of DNA was digested with *Sac* I, separated on a 0.8% agarose gel, and transferred onto the nylon membrane Hybond-N^+ (Amersham, Cleveland, OH). The radiolabeled DNA probe was a *neo* or GFP gene-specific sequence as described above.

RESULTS

Preparation of retroviral producer cells

The two retroviral plasmids pG1NGFP and pG1XGFP were modified from the parental plasmid pG1Na (Fig. 1) by the insertion of the S65T form of the GFP cDNA. Amphotropic retroviral packaging cells Ψ-CRIP and PA317 and ecotropic retroviral packaging cells Ψ-2 were transfected with the plasmids pG1Na, pG1NGFP, and pG1XGFP by the lipofectin method. Bulk producers and cloned producer cell lines expressing G1Na, G1NGFP, and G1XGFP were derived after G418 selection as described above in Materials and Methods.

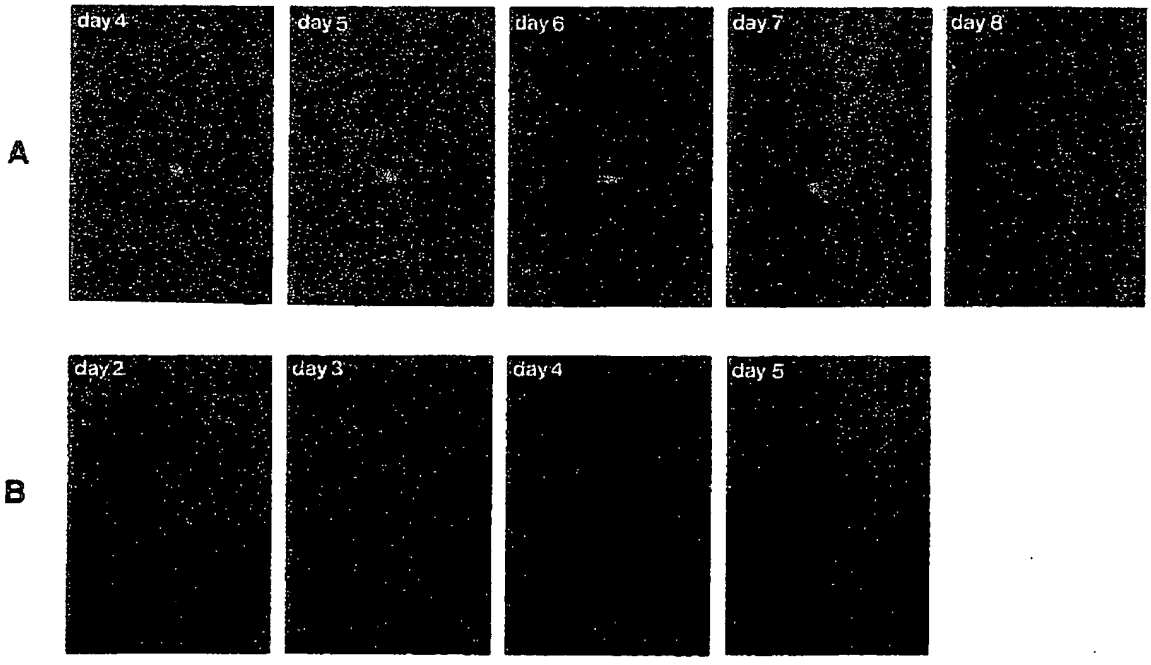

FIG. 2. Fluorescent microscopic observation of the Ψ-CRIP cells transfected with the GFP retroviral plasmid. Ψ-CRIP cells were transfected with pG1NGFP by the lipofectin method. Two days after transfection, the cells were placed in the presence of G418. Two typical examples (A and B) are shown here. Followed under confocal microscopy, the brightest cells were observed to form inclusion bodies and to contract and die within 7–10 days.

Observation of producer cells under fluorescent microscopy

The cells were followed beginning immediately after transfection for fluorescence using confocal microscopy. Fluorescence of cells transfected with the GFP-containing plasmids was observed for below 5% of the cells for the first 3–7 days of growth of the transfected cells, but by 10 days neither stable G418-selected clones nor bulk populations of cells fluoresced. The same loss of fluorescence was observed with or without G418 selection. Followed under confocal microscopy, the brightest cells were observed to contract and die within the first 7–10 days. Figure 2A shows an example of Ψ-CRIP cells transfected with pG1NGFP. A representative, very bright cell is shown at day 4 after transfection. The same cell had developed an inclusion body by day 7, and by day 8 the cell had contracted, died, and no longer fluoresced. Another example shows that two Ψ-CRIP cells transfected with pG1NGFP were fluorescing at day 2 after transfection (Fig. 2B). Some inclusion bodies were detected by day 4, and by day 5 the cells had died and no longer fluoresced. The same pattern of early fluorescence, inclusion body formation, and death of bright cells was seen with PA317, Ψ-2, and 3T3 cell lines transfected with the plasmids under the same conditions. No cells transfected with the control pG1Na plasmid ever fluoresced or showed similar patterns of inclusion body formation and cell death.

Virus production

Retroviral RNA was extracted from supernatants of the Ψ-CRIP producers, and slot-blot analysis was performed using a radiolabeled *neo* probe. The supernatants of the bulk G418-selected producers harvested 10–14 days after transfection were shown to contain low levels of viral RNA (Fig. 3A). There was little difference between amounts of viral RNA produced by bulk populations of cells transfected with pG1Na, pG1NGFP, or pG1XGFP. All were orders of magnitude lower than supernatant from the G1Na.40 clonal producer cell line used as a positive control.

The supernatants from individual producer cell clones selected by growth in G418 and harvested 4–6 weeks after transfection with the pG1Na plasmid were also shown to make viral RNA, although at levels 1–2 logs lower than the positive control producer G1Na.40 (Fig. 3B). However, no evidence of virus production from the pG1NGFP- or pG1XGFP-transfected clones could be obtained by slot-blot analysis (Fig. 3C). A total of 32 clones (9 clones for G1NGFP and 23 clones for G1XGFP) assayed had no detectable viral RNA production. We have repeated the slot-blot analyses looking at virus production from these clones with a GFP probe, and confirmed that no viral particles containing the GFP sequences were being produced (data not shown). We have also confirmed lack of virus production by these producer clones in a functional titering assay: 3T3 cells exposed to the viral supernatants from the G1NGFP or G1XGFP clones in the presence of protamine did not become G418-resistant, and did not show any fluorescence (data not shown).

Infection with transient GFP retroviruses

The BOSC23 cells were also used to test the retroviral constructs and to produce ecotropic retroviruses. We transfected

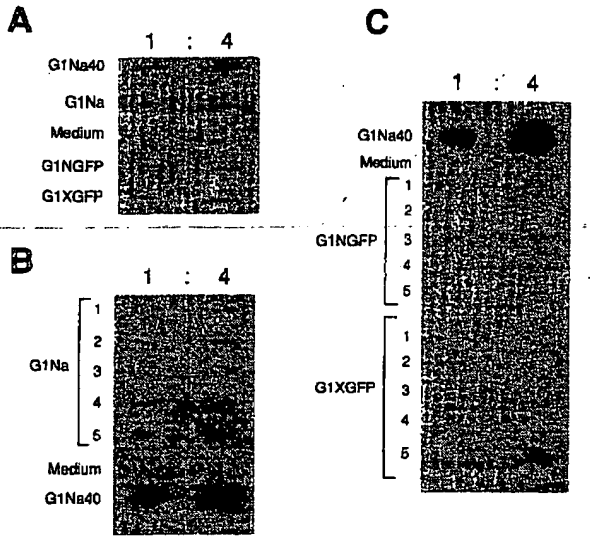

FIG. 3. Slot-blot analysis. Viral RNAs were extracted from 1 ml supernatants of producer cell line conditioned media. The RNAs were loaded into two adjacent wells in the ratio of 1:4 and transferred onto nylon membranes. The membranes were hybridized with a radiolabeled probe of the *neo* gene-specific DNA sequences. The positive control was the cloned producer cell line G1Na.40 supernatant (titer 1–5 × 10^6 particles/ml) and the negative control was the medium. The bulk producers (at 10–14 days after transfection) were shown to produce retroviruses of G1Na, G1NGFP, and G1XGFP (A). However, producer cell clones at 4–6 weeks after transfection for G1NGFP and G1XGFP and selection with G418 no longer produced viral RNA (C), although producer cell clones isolated at the same time containing G1Na still produced viral RNA (B).

the BOSC23 cells with our GFP plasmids by the calcium phosphate method. Two days after transfection, when approximately 80% of the cells fluoresced, the supernatants were harvested and filtered. RNA slot-blot probed with *neo* or GFP sequences revealed a high level of production of viral RNA, higher than a comparable producer cell line (G1Na.40) with a known biologic titer of 10^6 particles/ml (data not shown). The high percentage of cells visibly expressing high-level GFP was most likely due to the multiple copies per cell in the BOSC transiently expressing retroviral producer system. We then infected PA317 and Ψ-CRIP packaging cells with the supernatants. The number of the cells fluorescing was below 1%, probably reflecting the fact that most cells only contained a single copy of the vector, or a potential selective advantage favoring rearrangement of the vector in the transfected BOSC cells, with resultant poor transduction efficiency. The brightest cells infected with the virus died approximately 1 week after infection, even without G418 selection, in the same way as the packaging cells directly transfected with the GFP-containing plasmids.

Southern blot analysis of the producer cell lines

To try and explain the inability of the stable G418-resistant producer clones transfected with the GFP constructs to make retrovirus, Southern blotting was performed. Genomic DNA extracted from the Ψ-CRIP producer cell lines was digested with

Sac I, a restriction enzyme that cuts in the LTRs but not within the viral construct sequence. Each insertion of the plasmid sequences in the producer cell genome should result in 2.4-, 3.4-, and 3.6-kb bands for G1Na, G1NGFP, and G1XGFP, respectively, when probed with an internal probe such as *neo* gene sequences.

There were rearranged bands in four of the five G1NGFP clones studied and in four of five G1XGFP clones (Fig. 4A). Some clones were completely missing the band of the correct size (two of the five clones for G1NGFP, one of the five clones for G1XGFP). One clone of G1NGFP (clone 3) had a very high copy number of the inserted sequence in its genome, presumably due to duplication of the gene. We have reprobed the membrane with a GFP probe: Some bands disappeared, suggesting complete deletion of the GFP sequences, whereas other bands had the same pattern as with the *neo* gene probe, suggesting rearrangements in the viral regions (data not shown).

In the G1Na-producer cell clones derived at the same time, there was a significantly lower frequency of clones with rearranged bands of the incorrect size (Fig. 4B) as compared to either GFP construct. One clone had a larger rearranged band and was missing the correct band (clone 4). Nonetheless, the clone still produced viral RNA (Fig. 3C), indicating that the critical control and packaging sequences were intact. In total, there were 15 rearranged bands in the 10 GFP-producer cell lines, but at most two rearranged bands in the five G1Na-producer cell lines. We obtained similar results with Southern blotting of DNA from the Ψ-2 and PA317 clones (Table 1).

Producer cell lines expressing the humanized GFP

We have constructed pG1NhGFP in which the S65T-GFP gene was replaced by the humanized S65T-GFP (hGFP) gene, transfected the plasmid into Ψ-CRIP, Ψ-2, and PA317 cells, and established the producer cell lines in the same way as above. Early fluorescence, inclusion body formation, death of bright cells, and loss of fluorescence in the cloned producer cells were observed, similar to results with the other GFP genes. The Southern blotting of the humanized GFP-producer clones showed a high frequency of clones with rearrangements (17/33) comparable to that of the native GFP-producer clones (Table 1).

DISCUSSION

Other investigators have recently reported the use of wild-type GFP and more highly fluorescent variants as a genetic marker in retroviral vectors. In one study, retroviruses containing the GFP gene were obtained by transfections of BOSC23 cells (Cheng *et al.*, 1996) and only transient production of retroviruses containing GFP was documented. Another group has established stable producer cells (PA317) expressing the humanized GFP (Levy *et al.*, 1996). However, these producer cells were bulk producers followed for brief periods, not cloned cell lines that would typically be necessary for any preclinical or clinical applications.

We have attempted to isolate cloned stable producer cell lines producing high titer retroviral vectors containing the GFP gene. However, transfected packaging cells with high levels of GFP expression died within several days to weeks, as demonstrated by following individual cells via confocal microscopy. It is known that GFP can form inclusion bodies in *Escherichia coli* when it is expressed at high levels (Crameri *et al.*, 1996). We observed inclusion body formation prior to cell death in the transfected producer cells. Bulk-selected cells produced low-titer virus for short periods, but cloned cell lines were no longer fluorescent and no longer produced detectable vector RNA or biologically active viral particles. To exclude the possibility that direct transfection of the producer clones prevented successful vector production and stable fluorescence, the BOSC23 system

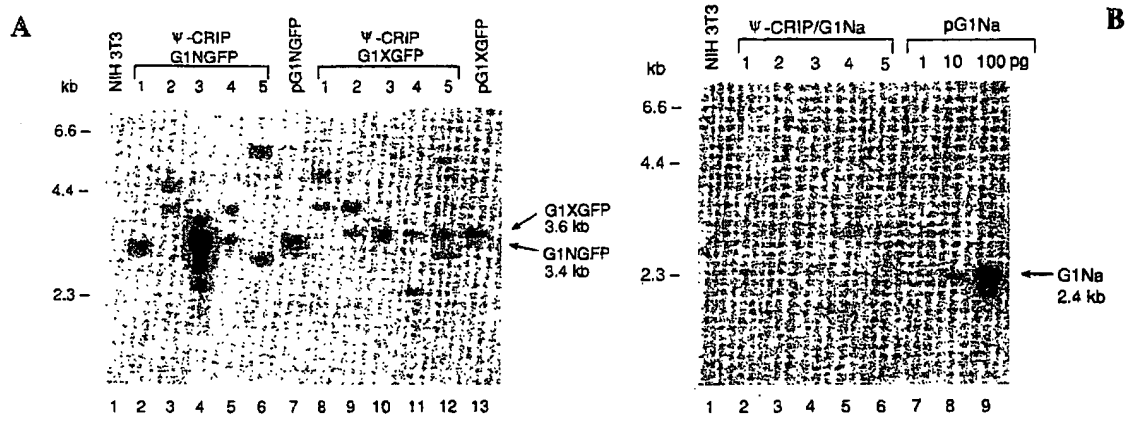

FIG. 4. Genomic Southern blotting of producer cell clones. GFP- (A) and G1Na-producer cell lines (B). Genomic DNAs (10 μg of DNA per lane) were extracted and treated with *Sac* I, which cuts in the LTRs but not within the viral construct sequences. The membranes were hybridized with a radiolabeled probe of the *neo* gene-specific DNA sequences. The plasmids, pG1NGFP (10 pg of the plasmid, lane 7 in A), pG1XGFP (10 pg of the plasmid, lane 13 in A), and pG1Na (1 pg of the plasmid, lane 7; 10 pg, lane 8; 100 pg, lane 9 in B) were used as the positive controls. Ten picograms of pG1NGFP and pG1XGFP corresponds to 1.6 copy per cell, and 10 pg of pG1Na corresponds to 2.5 copy per cell. Genomic DNA from NIH-3T3 cells was used as the negative control (lane 1 in A and lane 1 in B).

TABLE 1. REARRANGEMENT FREQUENCIES

	G1Na			*G1NGFP*			*G1XGFP*			*G1NhGFP*		
	a	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*
Ψ-CRIP	5	2	2	5	4	9	5	4	6	11	8	26
PA317	6	0	0	6	1	3	6	2	3	11	3	4
Ψ-2	4	0	0	6	4	5	6	3	3	11	6	10
Total	15	2	2	17	9[d]	17	17	9[e]	12	33	17[f]	40

a, number of clones examined; *b*, number of clones with rearranged bands; *c*, total number of rearranged bands.
[d]$p = 0.017$ (9/17 *vs.* 2/15).
[e]$p = 0.017$ (9/17 *vs.* 2/15).
[f]$p = 0.011$ (17/33 *vs.* 2/15).

was used to infect the same packaging cell lines, with similar loss of fluorescence and death of initially bright producer cells.

Clones that had rearranged the proviral genome in such a way that GFP expression was abrogated but that retained the *neo* gene necessary for selection appeared to have a strong advantage. This phenomenon was not seen in clones derived from transfection of packaging cells with non-GFP-containing vector sequences. The GFP-containing clones not shown to have gross DNA rearrangements of the provirus still did not produce viral particles. Presumably smaller-scale deletions, rearrangements, or even point mutations occurred in those cell lines that were not detectable by Southern blot analysis, but that nonetheless abrogated both GFP expression and virus production without abrogation of the *neo* gene expression.

The same problems were observed even when using humanized GFP, which was derived to take advantage of optimized human-type codon usage in hopes of improving translation (Levy *et al.*, 1996; Zolotukhin *et al.*, 1996). The base pair changes are all silent, thus the native and humanized GFP genes differ only in their nucleic acid sequences and have identical amino acid sequences. This suggests that the problem of selective disadvantage seems to be associated with expression of the GFP protein, not the gene itself or RNA expression.

It is less likely that the vector backbone itself becomes inherently unstable when expressing two transgenes. We have succeeded in establishing several producer cell lines containing the *neo* gene in the same position and expressing a second gene inserted into exactly the same cloning site as GFP, including G1NaIL3 (murine interleukin-3) and G1XmIFNγ (murine interferon-γ). Clones producing slot-blot-detectable, biologically active virus were obtained at high frequencies after G418 selection: 17/17 for G1NaIL3 and 32/40 for G1XmIFNγ (data not shown).

We have also transfected NIH-3T3, COS, and HeLa cells with non-retroviral plasmids containing S65T-GFP to establish stable cell lines expressing GFP. Despite initially fluorescent populations of cells, over time the cells were no longer fluorescent and similar cell death phenomena and inclusion bodies were observed under confocal microscopy. Thus, this phenomenon was not restricted to producer cells or to proviral plasmids. Transfected HeLa cells retained fluorescence for the longest period (2–3 weeks), possibly because HeLa cells are highly transformed and may be more resistant to the deleterious effects of GFP. There is one report of stable mammalian cell lines (BHK and CHO cells) expressing GFP (Olson *et al.*, 1995). But GFP was expressed as part of a fusion gene product with a cytoskeletal protein. This may have targeted the GFP to a location within the cell that was less problematic. Transgenic mice expressing the GFP gene have been reported (Ikawa *et al.*, 1995), but a systematic examination for any toxicity of the gene product has not been performed. Problems with GFP have been reported in other model systems. In plant cell transfections, it has been difficult to regenerate fertile plants from the brightest transfectants (Haseloff and Amos, 1995).

The lack of virus production by cell lines containing specific vector sequences is not limited to constructs containing the GFP gene. Similar phenomena have been observed in producer cell lines expressing oncogenes, including *abl* and *rel* (Pear *et al.*, 1993). Initially, the cell lines were shown to produce retroviruses at relatively high titers, but titers fell sharply with continued propagation of the cell lines. Although mechanisms were not well understood, it was hypothesized that it was due to some deleterious effect of the gene product (Ziegler *et al.*, 1981).

Recently, another form of mutant GFP was generated by using DNA shuffling to produce molecular evolution, with resulting 42-fold improvement in fluorescence over wild-type GFP (Crameri *et al.*, 1996). This new variant form of GFP may change the behaviors we have observed. Another possible approach would be to use an inducible promoter, allowing transient expression of GFP for a long-enough time to allow selection of transduced cells but perhaps brief enough to avoid significant toxicity. Currently, GFP is not suitable for inclusion as a selectable marker in the well-characterized vector/packaging systems utilized in this paper, but further understanding and modification of this unique protein may allow more successful applications.

REFERENCES

CHALFIE, M., TU, Y., EUSKIRCHEN, G., WARD, W.W., and PRASHER, D.C. (1994). Green fluorescent protein as a marker for gene expression. Science **263**, 802–805.

CHENG, L., FU, J., TSUKAMOTO, A., and HAWLEY, R.G. (1996). Use of green fluorescent protein variants to monitor gene transfer and expression in mammalian cells. Nature Biotech. **14**, 606–609.

CRAMERI, A., WHITEHORN, E.A., TATE, E., and STEMMER, W.P.C. (1996). Improved green fluorescence protein by molecular evolution using DNA shuffling. Nature Biotech. **14**, 315–319.

DANOS, O., and MULLIGAN, R.C. (1988). Safe and efficient gener-

ation of recombinant retroviruses with amphotropic and ecotropic host ranges. Proc. Natl. Acad. Sci. USA **85,** 6460–6464.

DUNBAR, C.E., and EMMONS, R.V.B. (1994). Gene transfer into hematopoietic progenitor and stem cells: Progress and problems. Stem Cells **12,** 563–576.

HASELOFF, J., and AMOS, B. (1995). GFP in plants. Trends Genet. **11,** 328–329.

HEIM, R., CUBITT, A.B., and TSIEN, R.Y. (1995). Improved green fluorescence. Nature **373,** 663–664.

IKAWA, M., KOMINAMI, K., YOSHIMURA, Y., TANAKA, K., NISHIMUNE, Y., and OKABE, M. (1995). Green fluorescent protein as a marker in transgenic mice. Develop. Growth Differ. **37,** 455–459.

KAIN, S., and GANGULY, S. (1995). Uses of fusion genes in mammalian transfection: Overview of genetic reporter systems. In *Current Protocols in Molecular Biology.* F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl, eds. (John Wiley & Sons, NY) unit 9.6.

LEVY, J.P., MULDOON, R.R., ZOLOTUKHIN, S., and LINK, C.J., JR. (1996). Retroviral transfer and expression of a humanized, red-shifted green fluorescent protein gene into human tumor cells. Nature Biotech. **16,** 610–614.

MANN, R., MULLIGAN, R.C., and BALTIMORE, D. (1983). Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. Cell **83,** 153–159.

MIGITA, M., MEDIN, J.A., PAWLIUK, R., JACOBSON, S., NAGLE, J.W., ANDERSON, S., AMIRI, M., HUMPHRIES, R.K., and KARLSSON, S. (1995). Selection of transduced $CD34^+$ progenitors and enzymatic correction of cells from Gaucher patients, with bicistronic vectors. Proc. Natl. Acad. Sci. USA **92,** 12075–12079.

MILLER, A.D., and BUTTIMORE, C. (1986). Redesign of retrovirus packaging cell lines to avoid recombination leading to helper virus production. Mol. Cell. Biol. **6,** 2895–2902.

OSLON, K.R., McINTOSH, J.R., and OLMSTED, J.B. (1995). Analysis of MAP4 function in living cells using green fluorescent protein (GFP) chimeras. J. Cell Biol. **130,** 639–650.

PEAR, W.S., NOLAN, G.P., SCOTT, M.L., and BALTIMORE, D. (1993). Production of high-titer helper-free retroviruses by transient transfection. Proc. Natl Acad. Sci. USA **90,** 8392–8396.

PRASHER, D.C., ECKENRODE, V.K., WARD, W.W., PRENDERGAST, F.G., and CORMIER, M.J. (1992). Primary structure of the *Aequoria victoria* green fluorescent protein. Gene **111,** 229–233.

ZIEGLER, S.F., WHITLOCK, C.A., GOFF, S.P., GIFFORD, A., and WITTE, O.N. (1981). Lethal effect of the Abelson murine leukemia virus transforming gene product. Cell **27,** 477–486.

ZOLOTUKHIN, S., POTTER, M., HAUSWIRTH, W.W., GUY, J., and MUZYCZKA, N. (1996). A "humanized" green fluorescent protein cDNA adapted for high-level expression in mammalian cells. J. Viol. **70,** 4646–4654.

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RESEARCH ARTICLE

Retroviral transfer and expression of a humanized, red-shifted green fluorescent protein gene into human tumor cells

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Over two-thirds of the current gene therapy protocols use retroviral gene transfer systems. We have developed an efficient retroviral-based method that allows rapid identification of gene transfer in living mammalian cells. Cells were generated containing a gene for an improved (humanized, red-shifted) version of the *Aequorea victoria* green fluorescent protein (hRGFP) from a retroviral vector. The hRGFP gene was used to produce an amphotropic vector producer cell line that demonstrated vibrant green fluorescence after excitation with blue light. A375 melanoma cells transduced with the retroviral vector demonstrated stable green fluorescence. Both PA317 murine fibroblasts and A375 human cell lines containing the vector were easily detected by FACS analysis. These vectors represent a substantial improvement over currently available gene transfer marking systems. Bright, long-term expression of the hRGFP gene in living eukaryotic cells will advance the study of gene transfer, gene expression, and gene product function in vitro and in vivo particularly for human gene therapy applications.

Keywords: retrovirus, green fluorescent protein, gene transfer, vector producer cell

Since the inception of DNA transfer technology, there has been an intense interest in gene marker systems that allow direct observation of transferred genes into living cells. Murine retroviral vectors have emerged in the past several years as the most common vehicle to deliver marker genes. Detection of markers such as β-galactosidase, luciferase, chloramphenicol acetyltransferase, and alkaline phosphatase involves either cell fixation that kills the cells or antibody-mediated detection. These methods are often time consuming and are prone to endogenous high background. Another group of gene transfer markers convey drug resistance and thus allow positive selection of transfected cells through selection of resistant colonies. Although drug selectable markers allow the detection of living cells expressing the transgene, they require that the cells survive in a toxic environment over a long period of time. These problems have led investigators to search for a better gene marker system that provides timely and accurate detection in living cells. One of the most promising new markers being developed to investigate gene transfer is the green fluorescent protein (GFP).

A number of species use a bioluminescent GFP to emit green light after energy transfer from either luciferases or photoproteins[1]. The GFP gene product can function as a marker in living cells and animals and does not require a substrate (other than light) to visualize gene transfer[2]. The GFP gene was cloned from the jellyfish *Aequorea victoria*, and the protein was found to have extremely stable fluorescence in vitro after stimulation with blue light[3].

The GFP cDNA encodes a 238 amino acid polypeptide with molecular weight of 27 kD[3]. GFP acts as an energy-transfer acceptor that under physiologic conditions in *A. victoria* receives energy from an activated aequorin-coelenterazine complex[4,5]. The chromophore is produced from autocyclization of three residues, serine-65, dehydrotyrosine-66, and glycine-67[4,6]. GFP is inactive until cyclization and oxidation of these three residues to generate a *p*-hydroxybenzylideneimadazolidinone chromophore (Fig. 1). Molecular oxygen is required for fluorophore formation[7]. The formed protein can be column purified, renatured, and crystallized and still maintain its fluorescent characteristics[8-10]. These results prompted expression studies of wildtype GFP in prokaryotic and eukaryotic cells[2]. This basic understanding of GFP mechanistic properties has led, in turn, to additional modifications to extend its usefulness to other systems. Recently, a gain of function mutant GFP gene was generated by Heim and colleagues[11] that altered the serine-65 codon to a threonine codon resulting in a red-shifted excitation peak. This red shifted GFP demonstrated superior fluorescence characteristics compared to wildtype GFP in prokaryotes[11].

A few investigators have humanized the wildtype codons[12]. *A. victoria* is classified in the phylum Ctenophora, and its codon usage is significantly different from that of mammals. Due to these differences, mammalian cells may not efficiently translate wildtype GFP mRNA. In this report, we describe the cloning and characterization of amphotropic retroviral vectors capable of demonstrating efficient, stable transfer of a humanized, red-shifted GFP (hRGFP) gene into mammalian cells. Retroviral vector-transduced living cells have a stable, bright green fluorescence after excitation with blue light. PA317 VPC or A375 melanoma cells expressing the hRGFP vector demonstrated an intense greenfluorescence by FACS analysis. These findings pave the way for a wide variety of experimental and clinical applications of modified GFP genes.

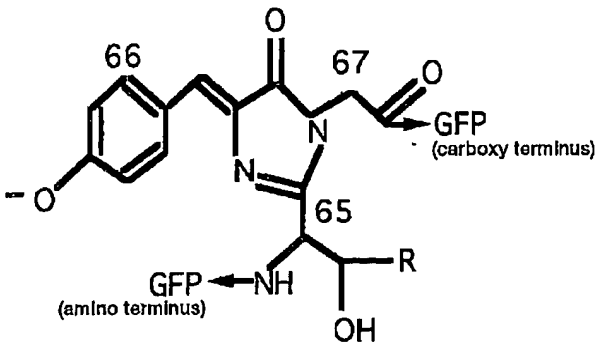

Figure 1. The GFP fluorophore. Blue light stimulates the excitation of a cyclized wildtype GFP fluorophore formed by serine-65, tyrosine-66, and glycine-67, emitting green fluorescence after stimulation. The red-shifted variant contains a mutation that converts serine-65 to threonine. This results in a red shift of the excitation wavelength, increased amplitude of fluorescence, and a faster rate of fluorophore formation in the mutant GFP[11]. R = H (serine); R = CH3 (threonine).

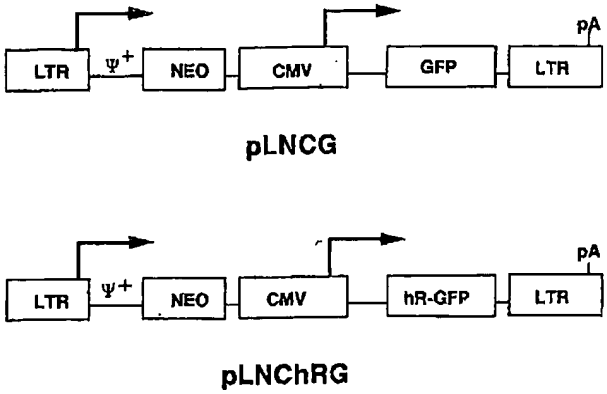

Figure 2. Retroviral constructs containing the red-shifted, humanized green fluorescent protein. The wildtype GFP and the humanized, red shifted GFP gene were cloned into the pLNCX retroviral backbone[19]. Plasmid pLNCG was constructed by subcloning a wildtype GFP DNA fragment into pLNCX. The LNChRG vector was constructed by subcloning a fragment containing the humanized, red-shifted GFP coding region into the multi-cloning site of pLNCX. (See Experimental protocol). LTR, long terminal repeat; pA, polyadenylation signal; arrows indicate transcriptional start sites; Ψ+ indicates the presence of the viral packaging sequence; GFP, wildtype green fluorescent protein; hRGFP, humanized, red-shifted GFP.

Results

Transfected cell lines. PA317 retroviral packaging cells and A375 melanoma cells were transiently transfected with pLNCG or pLNChRG plasmids (Fig. 2). These two constructs led to significantly different levels of fluorescence after transient transfection (Table 1). Nontransfected PA317 cells did not demonstrate green fluorescence (Fig. 3A). Transfected PA317 cells containing the wildtype GFP gene (pLNCG) exhibited fluorescence in <2% of the cell population that was detected after 48 h (Fig. 3B). However, transfection results with the humanized, red-shifted GFP retroviral construct (pLNChRG) were outstanding. Fluorescence can be detected as early as 9 h posttransfection. By 36 h, 30% to 40% or more of the cells are easily visualized, and contain enough protein to produce an intense fluorescence (Fig. 3D). Overall, with the fluorescence detection filters used, pLNChRG transfected PA317 cells had enhanced fluorescent intensity and efficiency compared to PA317 cells transfected with wildtype GFP plasmid (pLNCG). We did not observe any cytopathic or growth-inhibiting effect due to GFP or hRGPP in transfected cells.

Stable LNCG and LNChRG retroviral vector producer cells. Stable LNCG or LNChRG PA317 VPC were generated by lipofection. Transfected PA317 cells were selected in media containing G418 (1 mg/ml). These stable LNCG or LNChRG PA317 VPC were examined by fluorescence microscopy. The LNCG PA317 VPC line that contains the wildtype GFP gene exhibited no visible fluorescence after excitation with 420–470 nm light (data not shown). We therefore analyzed the LNCG VPC line by PCR using GFP amplimers to detect host chromosomal integration of the LNCG vector. The GFP gene was present in all lines tested (data not shown). In striking contrast, the LNChRG PA317 VPC line demonstrated vibrant green fluorescence in nearly 100% of the cells after G418 selection (Fig. 4). The fluorescence in the LNChRG VPC line was capable of highlighting many subcellular organelles.

Detection in LNChRG transduced A375 melanoma and NIH3T3tk- transduced cell lines. Supernatants from cultures of LNCG or LNChRG PA317 VPC were collected when the cells were 90% to 100% confluent. Supernatants were filtered and transferred to tissue culture plates containing A375 melanoma cells or NIH3T3tk- cells. Twenty-four hours after the final exposure to the retroviral supernatants, the target cells were placed under G418 selection. Most cells will contain only one integrated copy of the retroviral vector (C. Link, unpublished results). A375 cells transduced by LNCG VPC demonstrated no evidence of fluorescence despite the fact that PCR revealed the presence of GFP in the cellular genome (data not shown). However, the neo^r gene transferred by the LNCG vector was functional, since the A375 cell colonies were G418 resistant. In contrast, the LNChRG vector transduced A375 melanoma cells had bright fluorescence with nearly 100% of the selected cells demonstrating brilliant green fluorescent activity (Fig. 5A). Similarly, murine NIH3T3tk- fibroblasts transduced with the LNChRG retroviral vector demonstrated strong fluorescence in nearly 100% of the cells (Fig. 5B). A375 and NIH3T3tk- cells, which do not contain a transduced LNChRG, do not exhibit any detectable fluorescence (unpublished results). We did not observe any cytopathic or growth-inhibiting effect due to GFP or

Table 1. Cell lines expressing green fluorescent protein.

Target cell line	GFP gene transferred	GFP gene transfer method	Fluorescence intensity#	%Fluorescent cells@
PA317	GFP	Transient transfection	+	<2%
fibroblast	GFP	Stable transfection	0	0
	hRGFP	Transient transfection	+++++	30–40%
	hRGFP	Stable transfection	+++	>99%
A375	GFP	Transient transfection	+	<2%
melanoma	GFP	Stable retroviral transduction	0	0
	hRGFP	Transient transfection	+++	30–40%
	hRGFP	Stable retroviral transduction	+++	>99%

GFP: wildtype GFP gene without red-shift mutation or codon modifications; hRGFP: GFP gene modified to convert codon 65-serine to threonine and codon sequences modified to common mammalian usage. # Relative fluorescence intensity under examination by microscopy. @ Percentage of cells exhibiting fluorescence in the transfected or transduced population.

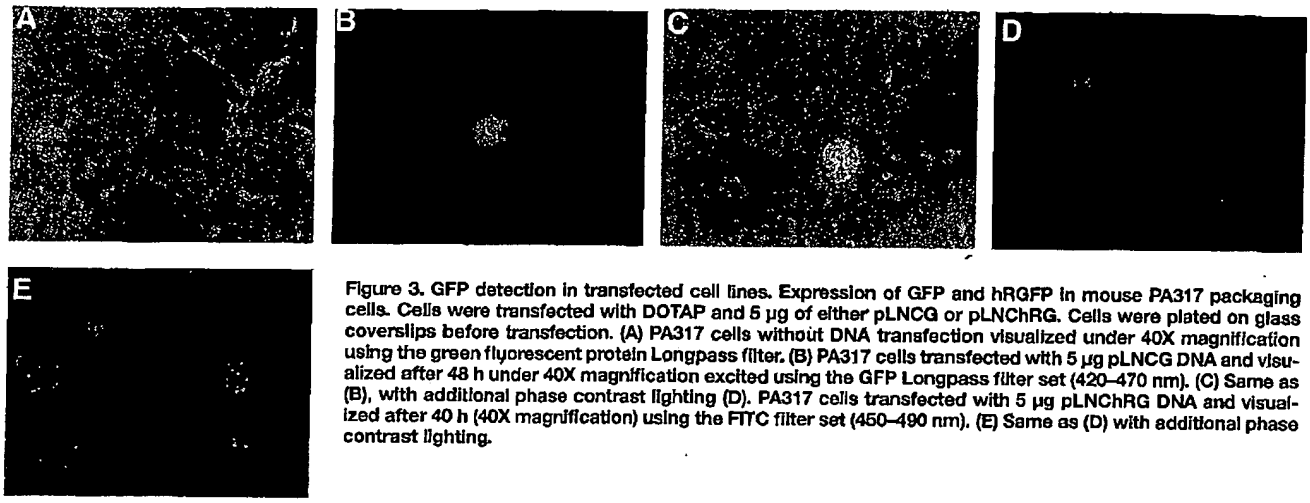

Figure 3. GFP detection in transfected cell lines. Expression of GFP and hRGFP in mouse PA317 packaging cells. Cells were transfected with DOTAP and 5 µg of either pLNCG or pLNChRG. Cells were plated on glass coverslips before transfection. (A) PA317 cells without DNA transfection visualized under 40X magnification using the green fluorescent protein Longpass filter. (B) PA317 cells transfected with 5 µg pLNCG DNA and visualized after 48 h under 40X magnification excited using the GFP Longpass filter set (420–470 nm). (C) Same as (B), with additional phase contrast lighting (D). PA317 cells transfected with 5 µg pLNChRG DNA and visualized after 40 h (40X magnification) using the FITC filter set (450–490 nm). (E) Same as (D) with additional phase contrast lighting.

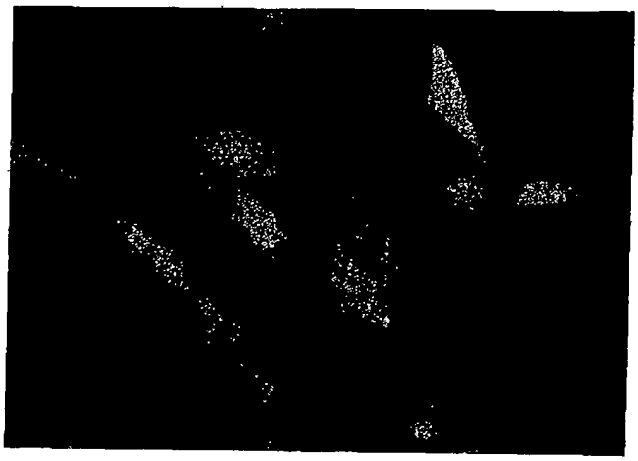

Figure 4. Detection of hRGFP gene activity in stable, transfected PA317 vector producer cells. Expression hRGFP in mouse PA317 packaging cell after transfection with pLNChRG and selection Selected cells were plated onto glass coverslips. PA317 cells transfected with pLNChRG were visualized >24 h after replating using the FITC filter set (40X magnification).

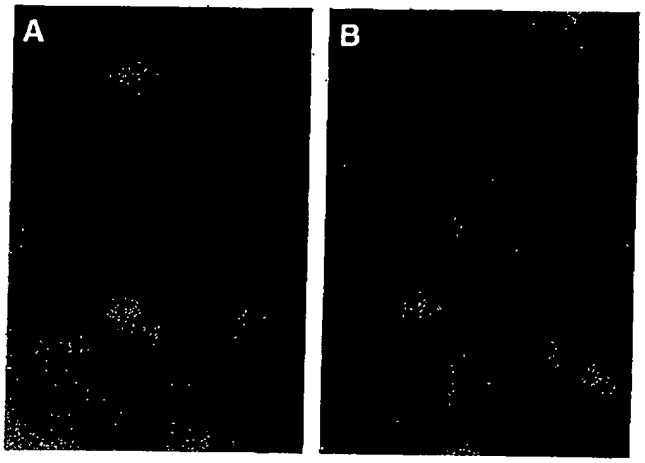

Figure 5. Expression of hRGFP in human A375 melanoma and murine NIH3T3tk- fibroblast cells after transduction with the LNChRG retroviral vector. G418 selected cells were plated onto glass coverslips and visualized using the FITC filter set. (A) A375 melanoma cells transduced with LNChRG retroviral vector (40X) magnification. (B) NIH3T3tk- murine fibroblast cells transduced with LNChRG retroviral vector (40X magnification).

hRGFP in transduced cells.

FACS analysis of GFP transfected PA317 vector producer cells and LNChRG transduced A375 melanoma cells. PA317 cells that had been transfected by the LNChRG vector and selected were analyzed by FACS. Excitation with 488 nm light resulted in light emissions at 525 nm in LNChRG-containing cells. PA317 cells transfected and expressing hRGFP (Fig. 6B) were easily detected by a two-log shift from nontransfected control PA317 cells (Fig. 6A). A375 melanoma cells transduced and selected with the LNChRG vector (Fig. 6D) were readily detected after excitation by shift in detected fluorescence over two logs compared to the control nontransduced A375 cells (Fig. 6C). These results demonstrate that GFP fluorescence can be quantified with available instrumentation.

Discussion

We have demonstrated the effectiveness of a humanized, red-shifted, mutant GFP by retroviral mediated gene transfer into human tumor cells and murine fibroblasts. Mutations of the wildtype GFP gene have resulted in GFP gene products with modified excitation and emission spectra[7,13]. The longer wavelength excitation peak (475 nm) of native *A. victoria* GFP has lower amplitude than its shorter wavelength excitation peak (395 nm)[7]. Heim and colleagues[11] used mutagenesis of the fluorophore to alter the serine-65 residue. They reported gain of function mutants—in which serine-65 was replaced with alanine, leucine, cysteine, or threonine—that show a single excitation peak (470–490 nm) with fluorescence amplitudes from fourfold to sixfold greater than the wildtype gene product. Interestingly, this mutant also shows a more rapidformation of the fluorochrome. We modified GFP such that it contained the red-shifted mutation and codons most commonly translated in mammals[12]. We have evaluated this humanized version of a serine-65 to threonine codon mutant that demonstrates emission at 510 nm in our current gene transfer experiments. Comparisons between the wildtype GFP and the humanized, serine-65 red-shifted mutant (hRGFP) demonstrated substantial improvement in fluorescence expression after either

transfection or retroviral-mediated GFP gene transfer (Table 1).

Wildtype GFP and GFP fusion proteins have been used in mammalian cells as markers of protein trafficking and gene expression[14–18]. Of note, these experiments used transient transfection to obtain very high levels of wildtype GFP expression. Our experiments are in agreement with these results in that transient transfection which transfers multiple transgene copies of wildtype GFP expression cassettes were easily visualized, but we found that stable transduced cells with a single transgene copy of wildtype GFP could never be visualized by fluorescence microscopy (Table 1). However, our results demonstrate that a humanized, red shifted GFP transgene in single copy can produce excellent fluorescence (Fig. 5). Transgenic mice have been produced expressing a slightly modified wildtype GFP gene expressed from the chicken β-actin promoter[19]. The fingers and tails of these transgenic mice were distinguishable as green under a fluorescent microscope, and homogenized tissue from the muscle, pancreas, lung, and kidney demonstrated fluorescence after excitation with 490 nm light. The visualization of vector gene expression in living transduced tissues with hRGFP may become an outstanding method to study in vivo gene transfer used in human clinical trials.

Experimental protocol

Cell culture. A375 is a human melanoma cell line (ATCC, Rockville, MD). PA317 is a murine amphotropic, retroviral vector packaging cell line (kindly provided by A.D. Miller, University of Washington, Seattle). NIH3T3tk- is a murine fibroblast cell line (kindly provided by Dr. Robert Goldberg, NIH/NCI). Cells were grown in RPMI supplemented with 10% fetal calf serum (FCS) (Gibco BRL, Gaithersburg, MD) in monolayers at 37°C and 5% CO_2. Retroviral vector producer cells (VPC) were grown in RPMI with 10% FCS in monolayers at 37°C and 5% CO_2. All cells were passaged and harvested by standard (Gibco BRL)digestion at 37°C. Cells were routinely passaged at 80% to 90% confluence.

Plasmid preparation and digoxin probes. pGFP-C1 containing wildtype GFP was obtained from Clontech (Palo Alto, CA). pTR-UF2 containing the hRGFP was kindly provided by Dr. Sergei Zolotukhin and Dr. Nicholas Muzyczka (University of Florida, Gainesville). Plasmid DNA was transformed into DH5α competent cells and colonies grown onL-broth supplemented with ampicillin (50 μg/ml) plates (LB/AMP) and transferred onto nylon membranes. The membrane was probed with aDig-GFP probe using a digoxin probe kit (Boehringer-Mannheim, Indianapolis, IN). Primers for the Dig-GFP probe amplification of a GFP fragment were 5′ primer 5′ GGG AAG CTT TTA TTA TTT GTA TAG TTC ATC CAT GCC and 3′ primer 5′ GGG AAG CTT GCG CGT ATG GGT AAA GGA GAA GAA CTT. Positive colonies were grown up in LB/AMP broth, and plasmid DNA was isolated using the Qiagen plasmid prep kits (Qiagen, Chatsworth, CA).

Construction of GFP retroviral vectors. Primers were made to amplify the 5′ end of the CMV promoter/enhancer and the 3′ end of the wildtype GFP gene from the GFP-C1 vector (Clontech). The 5′ primer includes unique XbaI, BamHI, and NotI restriction enzyme sites: 5′ GGA TCT AGA GGA TCC GCG GCC GCC TAG TTA TTA ATA GTA ATC AAT TAC GGG GTC. The 3′ primer includes 3 in-frame stop codons followed by a HindIII restriction enzyme site: 5′ GGA AAG CTT CTA TCA TTA TTG AGC TCG AGA TCT GAG TCC GGA CTT GTA. The 1.3 kb CMV-GFP PCR product was cloned into PCR3-cloning vector (Invitrogen, San Diego, CA) to generate plasmid pPCR3CG-14. The 1.3 kb fragment containing the CMV promoter and GFP gene was gel isolated (Jetsorb, Genomed, Bad Oeynhausen, Germany) from the pPCR3CG-14 vector using BamHI and HindIII restriction digest. The 800 bp retroviral CMV promotor was isolated from pLNCX using BamHI and HindIII and discarded. After gel isolation of the remaining 5.8 kb retroviral vector fragment, the 1.3 kb CMV-GFP insert was directionally cloned to generate the pLNCG construct. Finally, plasmid pTR_{ES}-UF2 (Gene Bank Accession # U50963) was restriction digested with NotI and the 730 bp DNA fragment containing the humanized red shift GFP open reading frame was isolated. After Klenow treatment, the blunt ended DNA was ligated into pLNCX at the HpaI site. The resulting plasmid was designated pLNChRG.

Fluorescent detection of green fluorescent protein expressing cells. We visualized GFP expressing cells with a Nikon Labophot-2 Fluorescent Microscope. The cube used in the microscope was either the green fluorescent protein Longpass 41015 filter set (excitation at 420–470 nm and emission at 490 to >600 nm) for the wildtype GFP detection (Chroma Technology Corporation) or the FITC dichromic filter set (excitation at 450–490 nm and emission at 520 nm) for the hRGFP detection. Photographs were taken using the Nikon Microflex UFX-DX and AFX-DX systems.

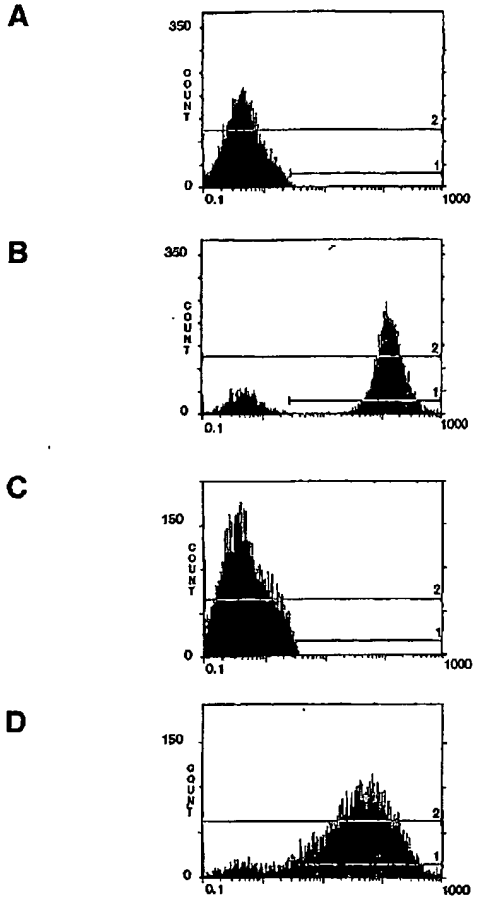

Figure 6. FACS analysis. Determination of hRGFP activity in stable populations of mammalian cells. Cells were trypsin-digested and washed before analysis in a EPICS Profile II Analyzer. (A) PA317 packaging cells without transfection (negative control). (B) LNChRG transfected PA317 vector producer cells expressing hRGFP after excitation. The large shift in peak detected mean fluorescence corresponds with hRGFP activity. (C) Nontransduced A375 cells (negative control). (D) LNChRG transduced A375 cells expressing hRGFP after excitation. The large shift in peak detected mean fluorescence corresponds with hRGFP activity. The FL1 emission channel was used to monitor green fluorescence. Count: cell number counted at given fluorescence intensity; y-axis: a log scale of mean intensity.

Transient expression of GFP. PA317 cells were seeded on a sterilized coverslip in a 6-well dish 12 to 24 h before transfection. Cells were at 30% to 50% confluence at the time of DNA transfection. Five μg of DNA and 15 μl of DOTAP reagent (Boehringer Mannheim) was used as per the manufacturer's protocol. The mixture was added to the plates containing either RPMI 1640 with 10% FBS, 1% L-glutamine, and penicillin/streptomycin or in serum free media. After 10 to 18 h the media was replaced with RPMI with 10% FCS. The coverslip containing the cells was placed on a slide and examined forfluorescence 9 to 48 h after transfection. The cells remaining in the well (after the coverslip was removed) were trypsin digested and transferred to tissue culture dishes. After attachment these cells were placed under selection with G418 (1 mg/ml) for 10 to 14 days.

Construction, subcloning, and titering of LNCG and LNChRG vector producer cell lines. The plasmid pLNCG or pLNChRG was transfected with DOTAP into the amphotropic retroviral packaging line PA317. Twenty-four hours later, the cells were placed under G418 (1 mg/ml) selection for 10–14 days. LNCG or LNChRG VPC were grown to approximately 90% confluence and supernatants were removed to transduce target cells. Retroviral supernatants were filtered through 0.45 μm filters (Nalgene, Kent, UK), supplemented with 10 μg/ml of protamine sulfate and used to transduce A375 melanoma or NIH3T3tk- fibroblast cells. The target A375 melanoma or NIH3T3tk- cells were 40% to 60% confluent when transduced. Twenty-four hours after the final transduction, cells were placed under G418 (1 mg/ml) selection for 10 to 14 days. Cells were examined by fluorescence microscopy after reseeding the cells onto glass cover slips.

Fluorescence activated cell sorter analysis of transduced human cells. Cytometry of stable hRGFP transfected or transduced cells was performed on a Epics Profile II Analyzer. Cells were analyzed using a 525 nm band pass filter set (Coulter Corp., Miami, FL). Cultures of nontransfected PA317 cells, LNChRG transfected PA317 cells, nontransduced A375 cells, or LNChRG transduced A375 cells that were 80% to 90% confluent were trypsin digested, washed with RPMI with 10% FCS, and resuspended at a concentration of approximately 1×10^6 cells/ml. All FACS analysis used the FL1 emission channel to monitor green fluorescence (normally a FITC monitor).

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1. Prasher, D.C. 1995. Using GFP to see the light. *Trends Genetics* 11:320–323.
2. Chalfie, M., Tu, Y., Euskirchen G., Ward, W.W., and Prasher, D.C. 1994. Green Fluorescent Protein as a marker for gene expression. *Science* 263:802–805.
3. Prasher, D.C., Eckenrode, V.K., Ward, W.W., Prendergrast, F.G., and Cormier, M.J. 1992. Primary structure of the *Aequorea victoria* green-fluorescent protein. *Gene* 111:229–233.
4. Cody, C.W., Prasher, D.C., Westler, W.M., Prendergast, F.G., and Ward, W.W. 1993. Chemical structure of the hexapeptide chromophore of the *Aequorea* green fluorescent protein. *Biochemistry* 32:1212–1218.
5. Inouye S. and Tsuji, F.I. 1994. *Aequorea* green fluorescent protein expression of the gene and fluorescence characteristics of the recombinant protein. *FEBS Lett.* 341:277–280.
6. Shimomura, O. 1979. Structure of the chromophore of *Aequorea* green fluorescent protein. *FEBS Lett.* 104:220–222.
7. Heim, R., Prasher, D.C., and Tsien, R.T. 1994. Wavelength mutations and posttranslational autoxidation of green fluorescent protein. *Proc. Natl. Acad. Sci. USA* 91:12501–12504.
8. Ward, W.W. and Bokman, S.H. 1982. Reversible denaturation of *Aequorea* green fluorescent protein: Physical separation and characterization of the renatured protein. *Biochemistry* 21:4535–4540.
9. Perozzo, M.A., Ward, K.B., Thompson, R.B., and Ward, W.W. 1988. X-ray diffraction and time resolved fluorescence analyses of *Aequorea* green fluorescent protein crystals. *J. Biol. Chem.* 263:7713–7716.
10. Deschamps, J.R., Miller, C.E., and Ward, K.B. 1995. Rapid purification of recombinant green fluorescent protein using the hydrophobic properties of an HPLC size-exclusion column. *Prot. Expres. Purific.* 6:555–558.
11. Heim, R., Cubitt, A.B., and Tsien, R.Y. 1995. Improved green fluorescence. *Nature* 373:663–664.
12. Zolotukhin, S., Potter, M., Hauswirth, W., Guy, J., and Muzyczka, N. 1996. A "humanized" green fluorescent protein cDNA adapted for high level expression in mammalian cells. *J. Virol. In press.*
13. Delagrave, S., Hawtin, R.E., Silva, C.M., Yang, M.M., and Youvan, D.C. 1995. Red-shifted excitation mutants of the Green Fluorescent Protein. *Bio/Technology* 13:151–154.
14. Rizzuto, R., Brini, M., Pizo, P., Murgia, M., and Pozzan, T. 1995. Chimeric green fluorescent protein as a tool for visualizing subcellular organelles in living cells. *Current Biol.* 5:635–642.
15. Kaether, C. and Gerdes, H. 1995. Visualization of protein transport along the secretory pathway using green fluorescent protein. *FEBS Lett.* 369:267–271.
16. Ogawa, H., Inouye, S., Tsuji, F.I., Yasada, K., and Umesono, K. 1995. Localization, trafficking, and temperature-dependence of the *Aequorea* green fluorescent protein in cultured vertebrate cells. *Proc. Natl. Acad. Sci. USA* 92:11899–11903.
17. Olson, K.R., McIntosh, R., and Olmstead, J.B. 1995. Analysis of MAP 4 function in living cells using green fluorescent protein (GFP) chimeras. *J Cell Biol.* 130:639–650.
18. Marshall, J., Molloy, R., Moss, G.W., Howe, J.R., and Hughes, T.E. 1995. The jellyfish green fluorescent protein: A new tool for studying ion channel expression and function. *Neuron* 14:211–215.
19. Ikawa, M., Kominami, K., Yoshimura, Y., Tanaka, K., Nishimune, Y., and Okabe, M. 1995. Green fluorescent protein as a marker in transgenic mice. *Develop. Growth Differ.* 37:455–459.
20. Miller, A.D. and Buttimore, C. 1986. Redesign of retrovirus packaging cell lines to avoid recombination leading to helper virus production. *Molec. Cell Biol.* 6:2895–2902.

RESEARCH ARTICLE

Use of green fluorescent protein variants to monitor gene transfer and expression in mammalian cells

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Two mutants of the green fluorescent protein (GFP), RSGFP4 and GFPS65T, have been recently created which differ from the wildtype GFP of *A. victoria* in their excitation maxima. Here we show that human fibroblasts transfected with either of the two mutant GFP genes emit a green fluorescence that is 18-fold brighter than the cells transfected with the wildtype GFP gene. Retroviral vectors expressing the improved GFP gene were also constructed to determine their suitability for stable gene transduction into mammalian cells. The inclusion of the RSGFP4 gene in a retroviral vector did not reduce the viral titer and resulted in a fluorescent signal in viable transduced cells detectable by both fluorescence microscopy and fluorescence-activated cell sorter (FACS) analysis. Therefore, the improved mutant GFP provides a vital marker for monitoring gene transfer and expression in mammalian cells.

Key words: GFP, FACS, fluorescence microscopy, gene transduction, gene therapy

The green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* has become an important marker of gene expression[1]. The monomeric GFP consists of 238 amino acids and requires no other *Aequorea* proteins, substrates, or cofactors to fluoresce[1-3]. Thus, it is superior to other reporters such as the *E. coli* lacZ enzyme which requires transport of a fluorogenic substrate across cell membranes, or to cell-surface epitope markers which require specific-antibody staining.

Detection of green fluorescence from the expressed GFP cDNA has been demonstrated in several heterologous systems including *E. coli*, *C. elegans*, and *D. melanogaster*[1-4]. However, the expression and detection of wildtype GFP (wtGFP) in mammalian cells reportedly failed[5]. Recently a fluorescent signal was detected by fluorescence microscopy when GFP-expressing vectors were transfected into cultured cells in which transgenes can be highly expressed[6,7] (unpublished data). Thus, the wtGFP expressed in cultured mammalian cells emits a detectable green fluorescent signal, albeit at a relatively low level.

The recombinant wtGFP purified from *E. coli*, as well as its native form, emits bright green light (λmax = 508 nm) when excited with an ultraviolet (UV) source (λmax = 395 nm), or fluoresce weakly when excited with a blue light (λmax = 470 nm). Excitation with a UV light rapidly diminishes GFP fluorescence whereas excitation with a 470 nm light results in weak but stable green fluorescence[1]. If the wtGFP expressed in mammalian cells displays the same excitation and emission spectra as in vitro, then use of a conventional fluorescence microscope or a fluorescence activated cell sorter (FACS) will not provide optimal excitation wavelengths for the wtGFP to achieve maximal emission signals. Fluorescence microscopes typically contain a bandpass filter transmitting a blue light (~480 nm) which is required to excite fluorescein and its derivatives to emit a green light (~530 nm). Common flow cytometers contain an Argon ion laser tuned at 488 nm, and at 350 nm (UV light) if a second laser is added. In both cases, the wavelengths of exciting light sources, around 488 nm or 350 nm, are significantly different from the GFP's 395 nm excitation maximum, and therefore are not optimal for detecting the wtGFP fluorescence signal.

Two GFP mutants with excitation maxima around 490 nm have been created. One such red-shifted mutant was created by replacing 3 amino acids[4]. This mutant GFP (RSGFP4) exhibits excitation and emission peaks at 490 nm and 505 nm, respectively. The other GFP mutant has a single amino acid substitution of serine–65 to threonine, GFPS65T[3]. The *E. coli*-expressed GFPS65T displays excitation and emission maxima at 490 nm and 511 nm, respectively, and is sixfold brighter than the wtGFP[3].

We show that the two mutant GFPs display a much brighter green fluorescence than the wtGFP in living mammalian cells. When examined by FACS, GFPS65T and RSGFP4 are 18-fold and 24-fold, respectively, brighter than the wtGFP. In addition, we have created retroviral vectors containing the improved GFP gene and used these vectors to express GFP in transduced cells.

Results

Analysis of wildtype and mutant GFP protein levels in transfected mammalian cells. To compare the wildtype and mutant GFP gene expression in mammalian cells, expression vectors containing either the wtGFP, RSGFP4, or GFPS65T were transfected into BOSC23 fibroblasts in which the transgene can be highly expressed. After two days, monolayers of transfected cells were examined by fluorescence microscopy with a fluorescein filter set (see Experimental protocol). Cells transfected with either of the two mutant GFP genes displayed much brighter green fluorescence than cells transfected with the wtGFP (data not shown). To quantitate the relative fluorescence intensities of these three GFP variants, transfected cells were analyzed by a flow cytometer. The GFPS65T and RSGFP4 expressed in BOSC23 cells are approximately 18-fold and 24-fold, respectively, brighter than the wtGFP, provided the expression levels of all the three GFPs are similar (Fig. 1). Similar results were obtained with COS-7 cells although a smaller fraction of COS-7 cells was transfected (data not shown). In addition, bright green fluorescent signals from the expressed GFPS65T and

RSGFP4 proteins (recorded in the fluorescein channel) did not spill over into the second emission channel, which is used to record other fluorophores with an emission wavelength longer than fluorescein (data not shown). This allows us to analyze or sort cells coexpressing GFP and another marker, using an appropriate fluorophore which fluoresces in the second channel. Likewise, we can analyze cells using a fluorescence microscope equipped with a dual bandpass filter set for two color analyses.

Since the emission maximum of RSGFP4 is measured at 505 nm in vitro, we investigated whether a 515/20 nm filter (which selectively transmits 505 to 525 nm emission light) would be better than the standard filter (530/30 nm), which is optimal for fluorescein emission. Using the RSGFP4-transfected BOSC23 cells, we observed little difference in signals between the two filters. However, the background green fluorescence displayed in mock-transfected cells was twofold to threefold lower when the 515/20 nm filter was used (data not shown). Similar observations were also made with other cultured cells such as PA317 murine fibroblasts. Therefore, the ratio of the RSGFP4 signal to noise (i.e., background cell green fluorescence) in a FACS analysis can be improved twofold to threefold if a 515/20 nm emission filter is used.

GFP expression via retroviral-mediated gene transduction. An improved GFP gene that can be detected rapidly and noninvasively would be an invaluable tool for defining gene transduction strategies. Since retroviral vectors are widely used for stable gene transfer, the RSGFP4 gene was inserted into the murine stem cell virus vector (MSCV) to examine the use of GFP as a reporter gene for retroviral-mediated gene transfer and expression. In this GFP-expressing vector (MGPN), expression of the RSGFP4 gene is controlled by the long-terminal repeat (LTR) while a selection gene (neomycin) is driven by an internal promoter (Fig. 2). The MGPN vector was transfected into the BOSC23 ecotropic packaging cell line, and cells were analyzed by FACS two days after transfection. Approximately 28% of the cells emitted a green fluorescent signal (Fig. 3B). Supernatant from the transfected producer cells was then collected and tested for viral production measured by neomycin gene transduction of NIH3T3 cells. The end-point titer of the MGPN and MSCV retroviruses was similar, ranging from approximately 0.5 to 1×10^6 G418-resistant colony forming units per milliliter (cfu/ml). These results indicate that the RSGFP4 gene is not detrimental to virus production.

In addition to measuring retroviral transduction of NIH3T3 cells by neomycin selection, we also examined GFP expression mediated by infection with the MGPN retrovirus. NIH3T3 cells as well as an amphotropic packaging line, PA317 murine fibroblasts, were infected with either MGPN or the control MSCV virus. The infected cells were then cultured for four days in the absence of G418 selection, before living cells were harvested and analyzed by FACS. More than 90% of MGPN-infected PA317 cells emitted green fluorescence compared to 0.5% for control MSCV-infected cells (Fig. 4). MGPN-infected cells yielded a positive population with a peak fluorescence 15-fold greater than background fluorescence of the PA317 packaging cells. Therefore, GFP can be stably expressed in mammalian cells via a retroviral-mediated gene transduction. The GFP fluorescent signal in transduced NIH3T3 cells is less significant, mainly due to the high background fluorescence of NIH3T3 cells (data not shown).

To determine whether viable GFP-expressing cells could be selected by FACS based on their fluorescent signal, we sorted out and subsequently expanded the top 10% of the MGPN-infected PA317 cells displaying the brightest fluorescence. More than 90% of sorted cells were viable and resistant to G418. After 7 days expansion in culture, sorted producer cells displayed a brighter (approximately fourfold) fluorescent signal than the unsorted cells (Fig. 5). In addition, the end-point virus titer, produced by the sorted cells (approximately 1.0×10^6 cfu/ml), increased fourfold over unsorted PA.MGPN producer (0.2×10^6 cfu/ml).

To coexpress the GFP gene with another gene of interest, an

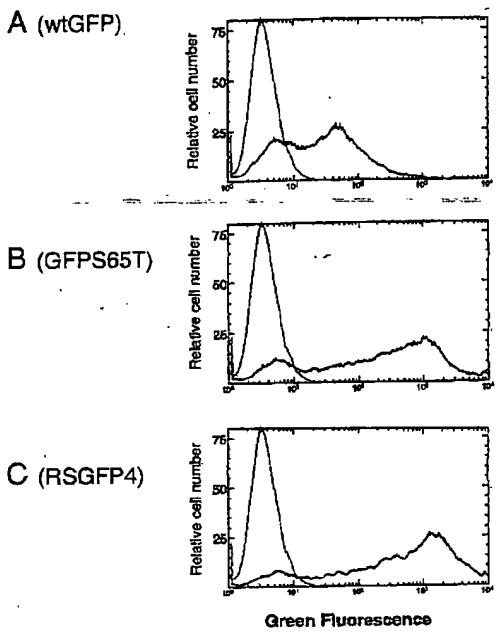

Figure 1. FACS analysis of GFP gene expression in transfected human cells. GFP-expressing plasmids containing either the wtGFP gene (A), GFPS65T (B), or RSGFP4 (C) were used to transfect BOSC23 cells. Forty hours post transfection, adherent cells were harvested and green fluorescence from transfected cells was analyzed by a flow cytometer tuned at 488 nm. The relative numbers of viable cells were then plotted as the function of variable intensities of green fluorescence from individual cells. The profile of mock-transfected cells (dotted lines) is overlaid for comparison. The relative intensities of the peak green fluorescence from cells transfected with pGFP-C1, pGFPS65T-C1 and pRSGFP-C1 are respectively 16-fold, 318-fold and 400-fold, greater than that of mock-transfected cells. Therefore, GFPS65T and RSGFP4 expressed in BOSC23 cells are approximately 18-fold and 24-fold, respectively, brighter than the wtGFP.

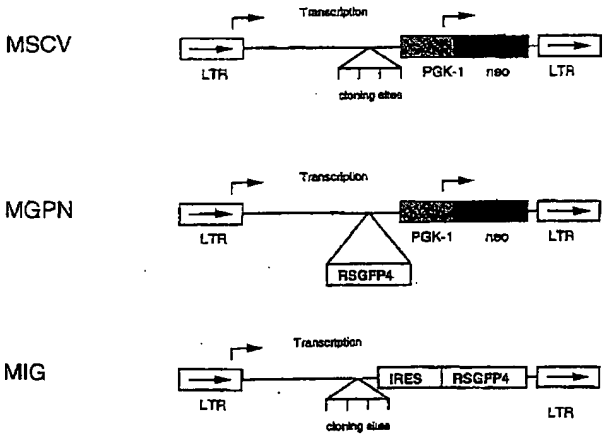

Figure 2. GFP-expressing retroviral vectors. Murine stem cell virus (MSCV) with a neomycin (neo) gene driven by an internal promoter PGK-1 is the parental vector. MGPN, the RSGFP4 gene is transcribed by the long-terminal repeat (LTR) of the MSCV. MIG, an internal ribosome entry sequence (IRES) was placed upstream of the RSGFP4 gene; the internal PGK-1 promoter and neo gene were deleted. See Experimental protocol for details.

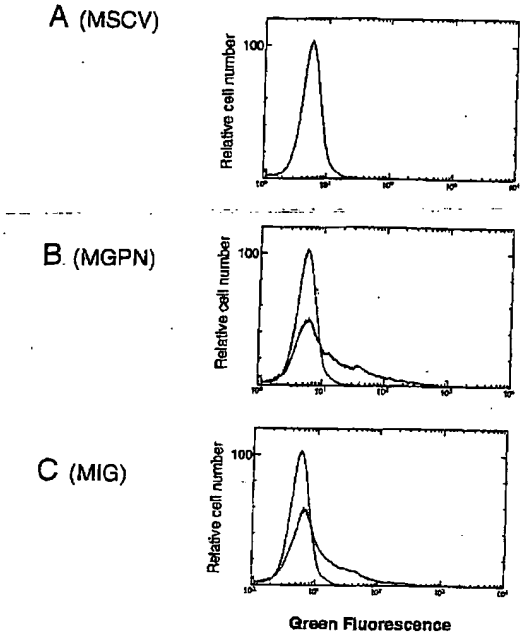

Figure 3. GFP expression in BOSC23 packaging cells transfected with retroviral vectors containing the RSGFP4 gene. BOSC23 cells were transfected with the MSCV (A), MGPN (B) or MIG (C) vector, and analyzed 2 days after transfection as in Figure 1. The profiles of cells transfected with MGPN (B) and MIG (C) are also overlaid with that of MSCV cells (dotted line). MGPN and MIG-transfected cells contain a cell population emitting green fluorescence (28% and 22.5% respectively) over the background (0.3% in MSCV-transfected cells).

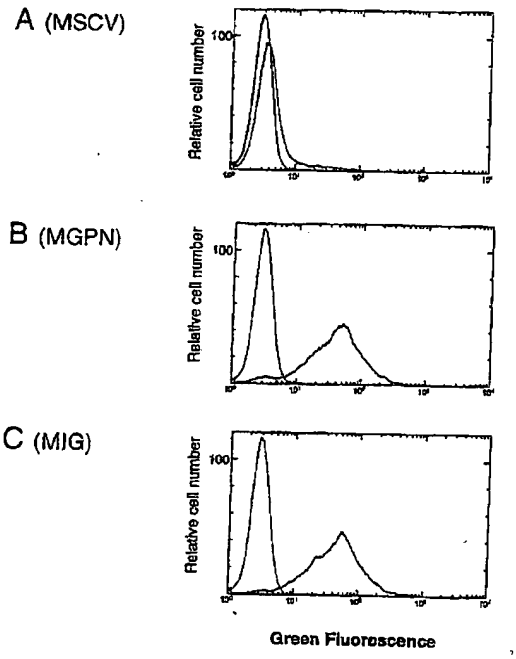

Figure 4. FACS analyses of GFP in transduced cells mediated by retroviral vectors. BOSC23 ecotropic packaging cells were transfected 3 days before the infection and viral-containing supernatants were subsequently collected. Then PA317 cells were then infected by either MSCV (A), MGPN (B) or MIG (C) viruses produced by BOSC23 producers, and analyzed 4 days after infection. The profile of mock-infected PA317 cells (dotted line) is overlaid. A 515/20 nm emission filter was used for the FL1 emission channel.

internal ribosome entry sequence (IRES) was placed upstream of the GFP gene in the MGPN vector after deleting the neomycin gene (Fig. 2). This construct creates two independent translational units, eliminating possible alterations in the GFP or the gene of interest resulting from the fusion of the two proteins. The IRES-containing GFP retroviral vector, MIG, displayed slightly weaker fluorescence in transfected BOSC23 packaging cells than the MGPN vector (Fig. 3C). MIG viruses produced by BOSC23 cells were then used to infect PA317 cells as before. The infected cells emitted a fluorescent signal similar to cells infected with the MGPN vector (Fig. 4C). Together these studies demonstrate that GFP variants such as RSGFP4 provide a simple method for selective cell sorting and isolation of high-titer viral producer clones based on the fluorescent signal. In addition, GFP variants can be coexpressed as a vital marker to monitor retroviral-mediated gene transfer and expression.

Discussion

We have analyzed the expression of wildtype and mutant GFP (GFPS65T and RSGFP4) in several types of mammalian cells and show that these proteins are stable and properly processed to form functional fluorophores. Expression of GFPs, either transiently or stably, are not detrimental to host cells. The GFPS65T and RSGFP4 are 18-fold and 24-fold, respectively, brighter than the wtGFP in mammalian cells as measured by flow cytometry. Therefore, these GFP variants are superior to the wtGFP for use with commonly used instruments optimized for fluorescein. In addition, the inclusion of RSGFP4 does not reduce the viral titer or the transduction efficiency of retroviral vectors. The improved GFP can be a useful marker either for monitoring gene transfer and expression, or for selecting transduced cells and high-titer viral producer clones.

A potential disadvantage of using GFP, over other reporter genes such as the lacZ gene, is its sensitivity limit. This could become critical in some applications in which the GFP gene can only be expressed at a low level. In these cases, GFP's green fluorescent signal may not be much over the background fluorescence of some target cells. Functional β-galactosidases (in tetrameric forms) encoded by the lacZ gene, in an hour can catalyze more than 10^5 substrate molecules, and prolonged incubation with excess substrates will further increase its maximal sensitivity[10]. However, the lacZ gene has some intrinsic limitations including: (1) endogenous β-galactosidase activities in some mammalian cells, (2) the requirements to transport fluorogenic substrates across cell membrane and to maintain the cleaved fluorescent products within viable cells, and (3) the enzymatic reaction time[10]. The latter two may limit the ability to sort viable cells expressing the lacZ gene, and prohibit real-time detection. In addition, the lacZ gene (3081 bp) is significantly larger than the GFP gene (710 bp), which may lead to reduced titers of retroviral vectors containing an additional gene of interest. Although we may not be able to reach the upper limit of sensitivity of the lacZ enzymatic assays, an improved GFP detection system and/or use of a further-improved GFP gene may constitute a more versatile reporter and vital marker that is sensitive enough for most biological applications.

We can envision several ways to further increase fluorescence sensitivity using GFP. One way is to place the improved GFP on the surface of cells to make it more accessible. Our preliminary experiments show that we can make a functional GFP associated with the outer cell membrane (data not shown). The localization of GFP on the cell membrane should also help the detection and imagery by fluorescence microscopy which is normally less sensitive than FACS. Alternatively, other GFP mutants known to be very bright in *E. coli*[11] may also be brighter in mammalian cells. Applications may include monitoring gene transfer and expression in gene therapy protocols, monitoring specific gene expression during critical developmental and disease states, and screening drugs which mod-

ulate the regulatory elements of gene expression.

Experimental protocol

DNA manipulations and expression vector constructions. Mammalian expression vectors containing either the wtGFP gene (pGFP-C1) or the RSGFP4 (pRSGFP-C1) were obtained from Clontech laboratories (Palo Alto, CA). In these vectors, the GFP genes are controlled by the immediate early promoter of human cytomegalovirus[6]. The vector containing the S65T point mutation was made by replacing the NdeI fragment (220 bp) in pGFP-C1 with a PCR-generated fragment containing a mutator primer based on the published sequence[9]. To make GFP-containing retroviral vectors, the 750 bp GFP fragment (from Eco47III to XhoI) from pRSGFP-C1 was cloned into the MSCVneoEB vector of the HpaI and XhoI sites[11]. The MSCVneoEB (MSCV) vector also contains a neomycin gene controlled by the internal PGK-1 promoter. This GFP-expressing retroviral vector is named MGPN. To create the retroviral vector MIG, in which the RSGFP4 is downstream to an internal ribosome entry sequence (IRES), the neomycin gene expression cassette (1300 bp) was first deleted from MGPN, and a 600 bp IRES fragment[12] was then inserted upstream to the RSGFP4 gene (the details of construction will be published elsewhere or provided upon request). All the plasmids were amplified in the DH5α *E. coli* strain (BRL, Gaitherburg, MD), and purified using a Promega's midi-preparation kit (Madison, WI).

Cell culture, transfection, fluorescence microscopy and FACS. Culture media (DMEM) were purchased from BRL and fetal calf serum (FCS) from Hyclone (Logan, UT). An ecotropic packaging cell line BOSC23, derived from the SV40 T antigen-transformed 293 human embryonic kidney (293T) fibroblasts, was cultured in DMEM plus 10% FCS[14]. The PA317 amphotropic packaging cells were cultured with DMEM plus 5% FCS[15]. For transfection, near-confluent BOSC23 cells were incubated with the precipitating mixture of CaPO, and saturating amounts of plasmids for 8 h before being replenished with fresh medium[14]. Unless otherwise indicated, BOSC23 cells were harvested by trypsin/EDTA (BRL) 40 h posttransfection, and resuspended in PBS plus 5 mM EDTA and 0.5% BSA for FACS analyses. Propidium iodide (0.5 μg/ml) was added to the cell suspension to exclude dead or dying cells from FACS analysis. Either a FACScan cytometer or FACStar^{plus} sorter (Becton-Dickinson, San Jose, CA) equipped with an Argon ion laser tuned at 488 nm was used, and green fluorescence is recorded in the FL1 emission channel (normally used to detect fluorescein or its derivatives). Unless otherwise indicated, a standard 530/30 nm filter is used for the FL1 emission channel. We have also compared a 515/20 emission filter with the 530/30 filter in the FACStar^{plus} sorter (all filters are made by Chroma Technology Corp., Brattleboro, VT). Cells infected by different GFP-containing vectors were analyzed similarly. An Olympus fluorescence microscope with a mercury arc lamp (100 Watt) and a fluorescein filter set (consisting of a 480/40 nm excitation filter and a 535/50 nm emission filter) was used to detect GFP in living cells, or fixed cells treated by 3.6% para-formaldehyde. Fluorescence in living cells cultured on plastic dishes (with full media) is readily detectable within 24 h after transfection with the GFP65T-or RSGFP4-expressing plasmid. The signal was further enhanced when the media was replaced by PBS.

Retroviral production and infection. The BOSC23 cells were also used to test the retroviral constructs and to produce ecotropic retroviruses[14]. Two days after transfection, confluent BOSC23 cells were incubated with a minimal volume of fresh medium at 32°C. Then supernatant containing virus was collected every 12 to 24 h over the next 72 h. The endpoint titers of viral stocks were measured based on the neomycin gene transduction in NIH3T3 cells (10 days in the presence of 1 mg/ml active G418 from BRL). Viral infection was done essentially the same as the published protocol[16]. Afterward cell pellets were resuspended in fresh medium and cultured for 2 to 5 days before being analyzed by FACS.

Note added in proof

Yang et al. also reported that by using fluorescence microscopy, they can detect green fluorescence from RSGFP4 and wildtype GFP in CHO-K1 mammalian cells transfected respectively with the pRSGFP-C1 and pGFP-C1 expression plasmids. Yang, T.-T., Kain, S.E., Kitts, P., Kondepudi, A., Yang, M.M., and Youvan, D.C. 1996. Dual color microscopic imagery of cells expressing the green fluorescent protein and a red-shifted variant. *Gene (in press)*.

Acknowledgments

We are grateful to Dr. Austin Smith for generously providing a plasmid containing the ECMV IRES fragment, Dr. Steven Kain for the pRSGFP-C1 vector, Dr. Carol Dammel for BOSC23 cells and Dr. Richard Rigg for PA317 cells. We are also thankful to Shirley Chen and Brian Ford for helping with FACS graphics, and Drs. Lishan Su, Beth Hill and David Van den Berg for their critical reading of the manuscript. RGH is supported by the National Cancer Institute of Canada.

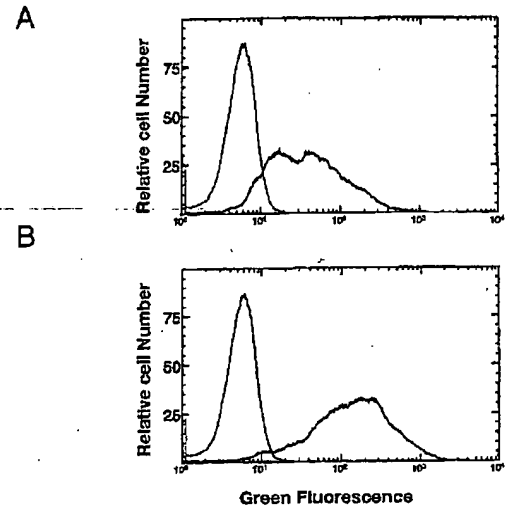

Figure 5. FACS analyses of GFP expression in stable PA317 packaging cells producing MGPN viruses. The PA317 packaging cells producing MGPN were generated by infection with BOSC23-produced MGPN viruses (see Fig. 4B). Part of these infected cells were used to establish a stable PA317 producer cell line (PA.MGPN) after G418 selection. The remainder were used to isolate the brightest 10% of GFP-expressing producers. After 7 days expansion in the presence of G418, both unsorted (A) and sorted (B) PA.MGPN producers were analyzed by FACS as described in Figure 1. The mean fluorescence intensity of sorted cells is fourfold greater than that of unsorted cells.

1. Chalfie, M., Tu, Y., Euskirchen, G., Ward, W.W., and Prasher, D.C. 1994. Green fluorescent protein as a marker for gene expression. *Science* 263(5148):802–805.
2. Cody, C.W., Prasher, D.C., Westler, W.M., Prendergast, F.G., and Ward, W.W. 1993. Chemical structure of the hexapeptide chromophore of the *Aequorea* green fluorescent protein. *Biochemistry* 32(5):1212–1218.
3. Inouye, S. and Tsuji, F.I. 1994. *Aequorea* green fluorescent protein. Expression of the gene and fluorescence characteristics of the recombinant protein. *FEBS Lett.* 341(2–3):277–280.
4. Wang, S. and Hazelrigg, T. 1994. Implications for bcd mRNA localization from spatial distribution of exu protein in *Drosophila* oogenesis. *Nature* 369(6479):400–403.
5. Stearns, T. 1995. Green fluorescent protein. The green revolution. *Curr. Biol.* 5(3):262–264.
6. Kitt, P., Adams, M., Kondepudi, A., Gallagher, D., and Kain, S. 1995. Green fluorescent protein (GFP): a novel reporter for monitoring gene expression in living cells and organisms. *Clontechniques* X(1):1–2.
7. Marshall, J., Molloy, R., Moss, G.W., Howe, J.R., and Hughes, T.E. 1995. The jellyfish green fluorescent protein: a new tool for studying ion channel expression and function. *Neuron* 14(2):211–215.
8. Delagrave, S., Hawtin, R.E., Silva, C.M., Yang, M.M., and Youvan, D.C. 1995. Red-shifted excitation mutants of the green fluorescent protein. *Bio/Technology* 13(2):151–154.
9. Heim, R., Cubitt, A.B., and Tsien, R.Y. 1995. Improved green fluorescence. *Nature* 373(6516):663–664.
10. Nolan, G.P., Fiering, S., Nicolas, J.-F., and Herzenberg, L.A. 1988. Fluorescence-activated cell analysis and sorting of viable mammalian cells based on β-galactosidase activity after transduction of *E. coli lacZ*. *Proc. Natl. Acad. Sci. USA* 85:2603–2607.
11. Cormack, B.P., Valdivia, R.H., and Falkow, S. 1996. FACS-optimized mutants of the green fluorescent protein (GFP). *Gene. In press.*
12. Hawley, R.G., Lieu, F.H.L., Fong, A.Z.C., and Hawley, T.S. 1994. Versatile retroviral vectors for potential use in gene therapy. *Gene Therapy* 1:136–138.
13. Ghattas, I.R., Sanes, J.R., and Majors, J.E. 1991. The encephalomyocarditis virus internal ribosome entry site allows efficient coexpression of two genes from a recombinant provirus in cultured cells and in embryos. *Mol. Cell. Biol.* 11:5848–5859.
14. Pear, W.S., Nolan, G.P., Scott, M.L., and Baltimore, D. 1993. Production of high-titer helper-free retroviruses by transient transfectiony. *Proc. Natl. Acad. Sci. USA* 90(18):8392–8396.
15. Miller, A.D. and Buttimore, C. 1986. Redesign of retrovirus packaging cell lines to avoid recombination leading to helper virus production. *Mol. Cell. Biol.* 6:2895.
16. Kotani, H., et al. 1994. Improved methods of retroviral vector transduction and production for gene therapy. *Hum. Gene. Ther.* 5(1):19–28.

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Genetics

Simultaneous fluorescence-activated cell sorter analysis of two distinct transcriptional elements within a single cell using engineered green fluorescent proteins

(genetics/transcription)

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Contributed by Leonard A. Herzenberg, April 9, 1996

ABSTRACT **Green fluorescent protein (GFP) is widely used as a reporter gene in both prokaryotes and eukaryotes. However, the fluorescence levels of wild-type GFP (wtGFP) are not bright enough for fluorescence-activated cell sorting or flow cytometry. Several GFP variants were generated that are brighter or have altered excitation spectra when expressed in prokaryotic cells. We engineered two GFP genes with different combinations of these mutations, GFP(S65T,V163A) termed GFP-Bex1, and GFP(S202F,T203I,V163A) termed GFP-Vex1. Both show enhanced brightness and improved signal-to-noise ratios when expressed in mammalian cells and appropriately excited, compared with wtGFP. Each mutant retains only one of the two excitation peaks of the wild-type protein. GFP-Bex1 excites at 488 nm (blue) and GFP-Vex1 excites at 406 nm (violet), both of which are available laser lines. Excitation at these wavelengths allows for the independent analyses of these mutants by fluorescence-activated cell sorting, permitting simultaneous, quantitative detection of expression from two different genes within single mammalian cells.**

The GFP gene, isolated from the jellyfish *Aequorea victoria*, encodes a protein that fluoresces upon excitation with violet or blue-green light. This gene is useful in both prokaryotes and eukaryotes as a reporter or marker (reviewed in refs. 1–3) and is unique among reporters in that the green fluorescent protein (GFP) fluorophore spontaneously forms intracellularly without added cofactors. As a result, the gene product provides a direct readout of gene expression, avoiding the difficulty of introducing a substrate into live cells and the complexity of enzyme-substrate kinetics.

Fluorescence can be detected by flow cytometry in mammalian cells transiently transfected with a wild-type GFP (wtGFP) expression vector. However, detection of fluorescence from cells containing a single copy of GFP has not been described. We find that the high levels of autofluorescence inherent in mammalian cells, combined with excitation spectra that are suboptimal for flow cytometry, precludes the detection of wtGFP when a single copy of the gene is stably integrated. Furthermore, mammalian cells are typically grown at 37°C, well above the temperature that yields maximum wtGFP fluorescence (4). The generation of mutant GFPs with improved stability and therefore increased fluorescence at 37°C, in combination with spectral changes optimized for fluorescence-activated cell sorting (FACS) analysis, would allow use of this reporter where low levels of expression are expected.

wtGFP has a major excitation peak at 395 nm, a minor excitation peak at 475 nm and a single emission peak at 509 nm (5). Several mutants were generated which retain one or the other of the excitation peaks when expressed in *Escherichia coli* (6–9). In addition, Bender *et al.* isolated a mutant (V163A) which retains the excitation and emission peaks of wtGFP but shows a 17-fold increase in fluorescence intensity (W. Bender, J. Kahana, A. Hudson, and P. A. Silver, personal communication) when it is expressed in *E. coli*. Presumably, this mutant forms a more stable GFP fluorophore in *E. coli* and other cells.

Dual laser FACS instruments equipped with krypton ion and argon ion lasers are capable of simultaneous excitations at 406 nm and 488 nm. In this study, we combine a mutant that excites primarily at 488 nm [S65T (7)] or a mutant which excites primarily at 406 nm [S202F, T203I (6)], with the V163A mutation, to generate two GFP variants, termed GFP-Bex1 and GFP-Vex1, respectively. Both variants show brighter fluorescence than the wild-type protein when expressed in mammalian cells. Fluorescence levels of both mutants are sufficient to detect GFP expression by FACS in cells with a single proviral integration. Thus mammalian cells can be viably sorted based on the level of GFP fluorescence, making these GFP mutants valuable genetic markers for mammalian studies. Furthermore, these mutants are spectrally distinguishable using FACS, allowing for the simultaneous, quantitative analysis of expression from two different promoters within a single cell.

MATERIALS AND METHODS

Plasmids, Mutagenesis and Sequencing. MFG-wtGFP-pBR322 was a kind gift from R. Mulligan. Analysis of MFG-wtGFP-pBR322 revealed a sequence differing from the published sequence (10) by the substitution of a G for A at nucleotide 239. This MFG-wtGFP-pBR322 backbone was mutagenized by PCR to generate a double mutant (S65T and V163A), termed GFP blue-excited excitation mutant 1 (GFP-Bex1) and a triple mutant (S202F, T203I, V163A) termed GFP violet-excited excitation mutant 1 (GFP-Vex1). The PCR-based site-directed mutagenesis strategy was as described by Picard *et al.* (11), with the addition of a Qiaquick Spin PCR Purification (Qiagen, Chatsworth, CA) step following the generation of the megaprimer. Subsequently, the coding fragments of mutant GFP variants from MFG-GFP-Bex1 and MFG-GFP-Vex1 were cloned into *Xba*I–*Hin*dIII digested pGL3 (Promega) to create pGL3-(GFP-Bex1) and pGL3-(GFP-Vex1). The tetracycline (tet) inducible construct, pGL3-UHD10-3-(GFP-Bex1) was created by inserting a modified fragment from pUHD10-3 (12) containing heptamerized tet operators into the *Xho*I–*Hin*dIII site of pGL3-(GFP-Bex1).

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Abbreviations: GFP, green fluorescent protein; wtGFP, wild-type GFP; FACS, fluorescence-activated cell sorter; tet, tetracycline; Bex1, blue-excited excitation mutant 1; Vex1, violet-excited excitation mutant 1; RSV, Rous sarcoma virus; IPTG, isopropyl β-D-thiogalactoside; moi, multiplicity of infection.

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The *Hin*dIII–*Sac*I fragment of pOPRSV1-CAT (Stratagene) containing the *lac* operator (*lac*O) and Rous sarcoma virus (RSV) promoter was inserted into pGL3-GFP(Vex1) upstream of the GFP coding region to create the *lac*-inducible construct pGL3-OPRSV1-GFP(Vex1). The *lac* repressor expression vector p3'SS and the tet repressor-vp16 chimera expression vector, pUHD15-1 were purchased from Stratagene. GFP coding regions were sequenced by dyedeoxy terminator cycle sequencing (PRISM Ready Reaction Kit; Perkin–Elmer). DNA sequence analysis was performed with IntelliGenetics software.

Cells, Tissue Culture and Retroviral Infection. NIH 3T3 cells (ATCC CCL 163) and BOSC 23 (13) and Phoenix retroviral producer cells (G.P.N., unpublished data) were maintained as described (13). Transfection of BOSC 23 and Phoenix producer cells as well as retroviral infection of 3T3 were as described (13). For gene induction experiments, BOSC 23 cells were maintained in 1 μg of tet per ml (Sigma) for 24 h and then transfected in the continued presence of tet. After 24 h, cells were harvested, split into four aliquots, and incubated for 48 h in the presence or absence of 1 μg of tet per ml and/or 50 mM isopropyl β-D-thiogalactoside (IPTG; Life Technologies, Gaithersburg, MD).

FACS Analysis. FACS analyses were carried out on a two-laser FACStar Plus platform (Becton Dickinson) modified by the Stanford FACS Development Group. An Innova 302 krypton ion laser (Coherent Radiation, Santa Clara, CA) tuned to 406 nm and an argon ion laser (Coherent Radiation) tuned to 488 nm were used in the dual laser experiments. To optimize detection of GFP fluorescence by FACS, the standard fluorescein filter was replaced with a 515/40-nm interference filter. Furthermore, to allow subtraction of the autofluorescence background signal, a detector with a 630/30 bandpass filter was used for autofluorescence compensation (14). On the FACS, the same emission fluorescence can be collected from two different laser excitations because the laser-stream intercepts are separated and the light emitted from each laser excitation follows a separate light path. Multiparameter data were collected and analyzed by using FACS-DESK configured as described (15). Where indicated, software compensation was applied to the collected data by using software from A. Treister (Stanford University, personal communication).

RESULTS

While wtGFP fluorescence can be detected by FACS in mammalian cells transiently transfected with the MFG-GFP plasmid [data not shown and Ropp *et al.* (16)], quantitative interpretation of these results is impossible due to the variable distribution of the reporter plasmid within a population of transfected cells. To determine whether the GFP expressed from a single GFP gene per cell fluoresces at levels sufficient for detection by FACS, retroviral gene transfer (13) was used to stably incorporate the MFG-wtGFP construct in NIH 3T3 cells. Using 488-nm excitation, FACS analysis of MFG-wtGFP transduced cells revealed a single peak on a fluorescence histogram (Fig. 1*B*). While the median fluorescence value of the MFG-wtGFP-infected population was 2-fold greater than that of uninfected cells (Fig. 1*A*), the difference in fluorescence was not sufficient to resolve infected from uninfected cells.

We next transduced a new GFP variant, including the S65T and V163A mutations (termed GFP-Bex1) under conditions yielding infection efficiencies comparable to those achieved with the MFG-wtGFP (data not shown). In contrast to the results found with wtGFP, FACS analysis of NIH 3T3 cells infected with MFG-GFP-Bex1 revealed two populations of cells (Fig. 1*C*), one with fluorescence levels indistinguishable from uninfected cells, and the second with a median fluorescence ≈50-fold greater than uninfected cells.

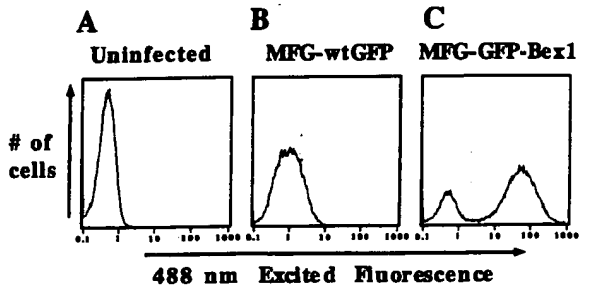

FIG. 1. Comparison of the wtGFP and GFP-Bex1 fluorescence levels in mammalian cells. NIH 3T3 cells infected with wtGFP or GFP-Bex1 retrovirus were analyzed 48 h after infection. Shown is fluorescence from 488-nm excitation of (*A*) uninfected NIH 3T3 cells, (*B*) MFG-wtGFP, or (*C*) MFG-GFP-Bex1-infected cells. This fluorescence was collected at 495–535 nm and is displayed as a histogram. Representative plots from three independent experiments are shown.

The latter population of cells exhibited 25-fold greater fluorescence than cells infected with wtGFP (compare Fig. 1 *B* to *C*), an increase sufficient to allow for the complete resolution of infected from uninfected cells. This population represents 60% of the total cells. Poisson statistics predicts that the probability of an infected cell carrying only a single viral integrant is ≈0.36. As the fluorescence levels of all of the GFP positive cells are distinguishable from nontranduced cells, this mutant can be used to accurately measure infection efficiencies.

The fluorescence levels achieved with GFP-Bex1, and the narrow excitation profile (with a peak at 488 nm) of the S65T mutation when expressed in *E. coli* (7), prompted us to explore the possibility of establishing a second GFP mutant with a distinct excitation spectrum for use on the FACS. To show that GFP-Bex1 is excited primarily with 488-nm light when expressed in mammalian cells, we configured a dual laser flow cytometer to excite cells at 488 nm and 406 nm, successively, and measure the emission at 495–535 nm in both cases (described in the *Materials and Methods*).

FACS analysis with 488-nm excitation reveals that 3T3 cells infected with MFG-GFP-Bex1 at a 5-fold lower multiplicity of infection (moi) than used in Fig. 1 show 40-fold greater fluorescence than uninfected cells, whereas excitation with 406-nm light yields fluorescence levels only 3-fold greater than uninfected cells (Fig. 2*A*). The low level of spectral overlap can be corrected by using software compensation, which corrects for the 406-nm excited fluorescence component of GFP-Bex1 from the 488-nm excitation value (compare Fig. 2 *A* and *B*).

The low level of intrinsic 406-nm excitation exhibited by GFP-Bex1 prompted us to establish a second composite GFP variant that has high excitation at 406 nm but minimal excitation at 488 nm, for use in combination with GFP-Bex1 on the FACS. The double-mutant GFP (S202F, T203I) was previously shown to be maximally excited at 395 nm, with only minimal excitation at 488 nm (6). To increase the fluorescence intensity of this mutant, we added the V163A mutation to generate the triple mutant MFG-GFP (S202F, T203I, V163A), termed GFP-Vex1.

We produced MFG-GFP-Vex1 retrovirus and infected 3T3 cells as before. FACS analysis revealed that excitation of cells with 406-nm light results in 16-fold greater fluorescence levels than those found in uninfected cells. In contrast, 488-nm excitation of GFP-Vex1 results in only 3-fold greater fluorescence levels (Fig. 2*C*). This low level of 488-nm excited fluorescence can be corrected for by software compensation, as shown in Fig. 2*D*.

The distinct spectral properties of GFP-Vex1 and GFP-Bex1 indicates that these fluorophores can be used in combination for dual reporter analyses. To confirm that these variants can be detected simultaneously within a single cell, we infected

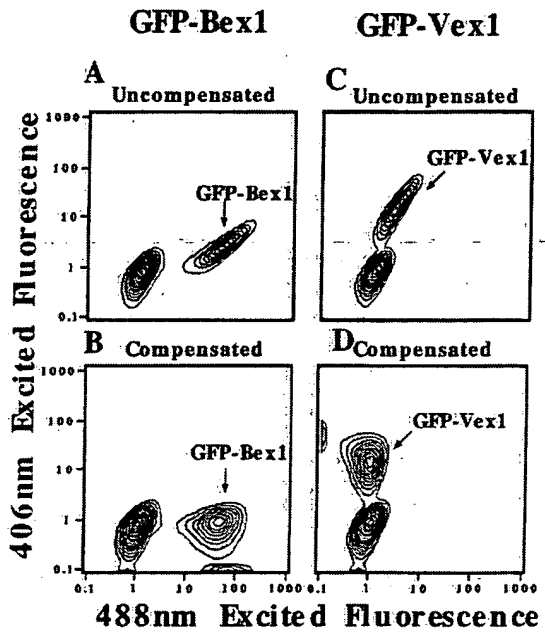

FIG. 2. Raw and compensated data of GFP-Bex1 and GFP-Vex1 fluorescence in NIH 3T3 cells. NIH 3T3 cells were infected with MFG-GFP-Bex1 (*A* and *B*) or MFG-GFP-Vex1-GFP (*C* and *D*) retrovirus and analyzed by flow cytometry 48 h postinfection. The moi was ≈20% of that used in Fig. 1. Emission data collected with 406-nm and 488-nm excitation are displayed on the abscissa and ordinate, respectively, in uncompensated (*A* and *C*) and compensated (*B* and *D*) form. Representative plots from three independent experiments are shown.

NIH 3T3 cells with equal amounts of GFP-Vex1 and GFP-Bex1 virus at three different mois (Fig. 3). FACS analysis of cells infected at an moi of 0.065 for each variant reveals three populations of cells: one with low levels of fluorescence characteristic of uninfected cells, and two with higher levels of fluorescence (Fig. 3*A*). The latter, clearly distinguishable populations were excited exclusively with 488- or 406-nm light (Fig. 3*A*), consistent with infection by a single retrovirus of either the GFP-Bex1 or GFP-Vex1 type, respectively.

Infection with 2-fold more retroviral supernatant yields the predicted moi of 0.13 for each virus, and generates, in addition to the two single positive populations shown in Fig. 3*A*, a new "double positive" population showing bright fluorescence with both 488-nm and 406-nm excitation (Fig. 3*B*), which is consistent with cells expressing both GFP variants due to coinfection. With a 5-fold further increase in the amount of retroviral supernatant used for infection, the number of double positives increases. These results are consistent with those predicted by the Poisson distribution with an moi of 0.65.

We next tested whether the two GFP variants can be used simultaneously as reporters of two distinct regulatory elements. We developed a tet transactivator controlled GFP-Bex1 expression system and a *lac* repressor controlled GFP-Vex1 expression system for this purpose. Cotransfection of a vector with GFP-Bex1 under the control of the tet operator (12) with a second plasmid, pUHD15-1, encoding the tet transactivator (a chimera of the tet repressor and the VP16 activation domain) leads to the induction of GFP-Bex1 expression. However, in the presence of tet, the tet activator dissociates from the tet operator, repressing transcription of GFP-Bex1 (data not shown).

Transfection of a plasmid encoding GFP-Vex1 under the control of the *lac* operator and RSV promoter (17) with p3'SS, a plasmid constitutively expressing a modified *lac* repressor, leads to the repression of GFP-Vex1 expression. However, in the presence of IPTG, the *lac* repressor–DNA complex is disrupted, thereby inducing transcription of GFP-Vex1 (data not shown).

We cotransfected BOSC 23 cells with all four plasmids simultaneously, divided the cells into four populations, and tested the effects of inducing one, the other, both, or neither forms of GFP by the addition or removal of tet and/or IPTG. Examination of the two-dimensional contour and histogram plots reveals that the fluorescence generated with 488-nm excitation increases only for the populations cultured under inducing conditions for GFP-Bex1 (i.e., −tet) (Fig. 4 *B* and *D* versus *A* and *C*). This increase is not influenced by inducing conditions for GFP-Vex1 expression (i.e., +IPTG) (Fig. 4*D*). In contrast, the fluorescence generated with 406-nm excitation increases only for the populations cultured under inducing conditions for GFP-Vex1 (i.e., +IPTG) (Fig. 4 *C* and *D* versus *A* and *B*). This increase is not influenced by inducing conditions for GFP-Bex1 expression (i.e., −tet) (Fig. 4*D*). Induction of GFP-Bex1 and GFP-Vex1 simultaneously (i.e., −tet, +IPTG) increases the fluorescences at both 406-nm and 488-nm excitation (Fig. 4*D*).

Under GFP-Bex1 inducing conditions, the median fluorescence of the transfected population excited at 488 nm increased ≈10-fold, while fluorescence excited at 406 nm (GFP-Vex1) showed no increase. In contrast, under inducing conditions for GFP-Vex1, fluorescence excited at 406 nm increased 2-fold, with no increase in fluorescence excited at 488 nm (GFP-Bex1). Based on these quantitative and qualitative comparisons, we conclude that GFP-Bex1 and GFP-Vex1 can be analyzed independently on the FACS, allowing for the simultaneous analysis of two distinct regulatory elements controlling these reporter genes.

DISCUSSION

Our studies show that GFP-Bex1 and GFP-Vex1 have sufficient fluorescence signal to be readily detected by FACS, and that both variants can be quantitatively detected indepen-

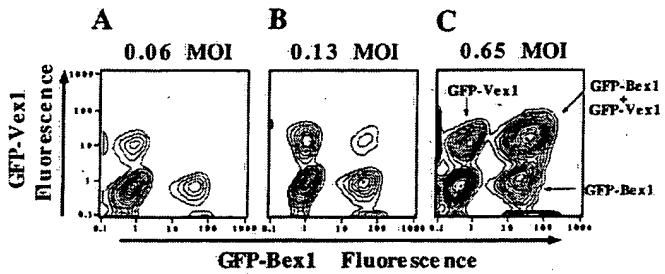

FIG. 3. Simultaneous detection of GFP-Bex1 and GFP-Vex1 fluorescence. Increasing amounts of a 1:1 mixture of the GFP-Bex1 and GFP-Vex1 viruses were used to infect NIH 3T3 cells. Cells were infected with an moi for each of the two viruses of 0.06 moi (*A*), 0.13 moi (*B*), or 0.65 moi (*C*), as determined by multiplying the moi measured in *A* by the relative amount of supernatant used in *B* and *C*. FACS analysis was conducted as described for Fig. 2 *B* and *D*. Representative plots from three independent experiments are shown.

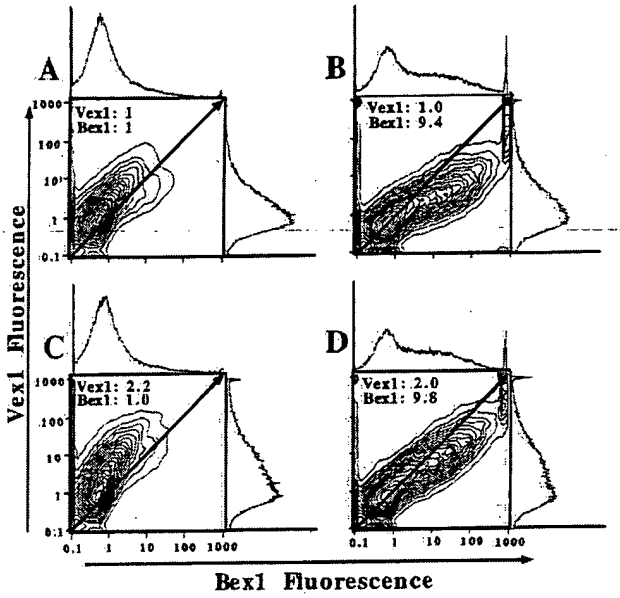

FIG. 4. Simultaneous analysis of two independently inducible transcription elements. BOSC cells were transiently transfected with the *lac*-inducible construct pGL3-OPRSV1-(GFP-Vex1), the tet inducible construct; pGL3-UHD10-3-(GFP-Bex1), the tet transactivator expression plasmid pUHD15-1; and the *lac* repressor expression plasmid p3'SS in the presence or absence of tet and/or IPTG, as described. After 48 h of culture under inducing and/or noninducing conditions, transfected BOSC cells were analyzed on the FACS as described in Fig. 2 *B* and *D*. Contour and histogram plots of uninduced GFP-Bex1 (*A* and *C*) and induced GFP-Bex1 (*B* and *D*) transduced cells under each culture condition are shown. The populations represented in *C* and *D* were also cultured under GFP-Vex1-inducing (+IPTG) conditions. The diagonal line through each contour plot is included as a visual reference. We calculated that ≈70% of the transfected cells were transduced by comparing the fluorescence histograms of mock transfected and transfected cells under inducing conditions. The 65th percentile fluorescence value of each population thus represents the median fluorescence of successfully transduced cells. To calculate the fold increase in fluorescence, these values were normalized to those obtained for the population cultured in the absence of inducing conditions depicted in *A*. These values are displayed in the upper left corner of each contour plot. Data representative of three independent experiments is shown.

dently, permitting their simultaneous detection at the single cell level. As GFP(S65T) and GFP(V163A) are only 5- to 6-fold brighter than wtGFP when expressed in 3T3 cells (data not shown), the 25-fold increase in brightness achieved with the composite GFP(S65T,V163A) fluorophore, GFP-Bex1, reflects a synergy of the S65T and V163A mutations. We use a FACS with a krypton ion laser for excitation at 406 nm for exciting the GFP(S202F,T203I) fluorophore. Unfortunately, this GFP is not significantly brighter than wtGFP when excited at 406 nm (6). However, the addition of the V163A substitution generates a GFP (GFP-Vex1) that is bright enough when excited with the krypton ion laser at 406 nm to yield a signal-to-noise ratio sufficient for quantitative analysis.

The two GFPs used in our studies differ in sequence by only 3 amino acids. Because of this structural similarity, these variants should be very similar in their transcription, translation, and protein stability characteristics. Therefore, the levels of fluorescence of GFP-Bex1 and GFP-Vex1 can be used to accurately determine the relative activity of two independent transcriptional elements by FACS.

The cotransfection experiments described here establish that GFP reporter genes can be used to quantitatively study gene regulation from two different gene regulatory elements. Recently, Rizzuto *et al.* (18) demonstrated that two different GFPs could be qualitatively distinguished within single cells by fluorescence microscopy, adding to the potential uses of the GFPs described here. Thus, GFP-Bex1 and GFP-Vex1 should be useful tools for dissecting signal transduction pathways, determining hierarchies of gene expression and in marking cells for genetic complementation.

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1. Peters, K. G., Rao, P. S., Bell, B. S. & Kindman, L. A. (1995) *Dev. Biol.* **171**, 252–257.
2. Rizzuto, R., Brini, M., Pizzo, P., Murgia, M. & Pozzan, T. (1995) *Curr. Biol.* **5**, 635–642.
3. Cubitt, A. B., Heim, R., Adams, S. R., Boyd, A. E., Gross, L. A. & Tsien, R. Y. (1995) *Trends Biochem. Sci.* **20**, 448–455.
4. Ogawa, H., Inouye, S., Tsuji, F. I., Yasuda, K. & Umesono, K. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 11899–11903.
5. Ward, W. W., Cody, C. W., Hart, R. C. & Cormier, M. J. (1980) *Photochem. Photobiol.* **31**, 611–615.
6. Heim, R., Prasher, D. C. & Tsien, R. Y. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 12501–12504.
7. Heim, R., Cubitt, A. B. & Tsien, R. Y. (1995) *Nature (London)* **373**, 663–664.
8. Heim, R. & Tsien, R. Y. (1996) *Curr. Biol.* **6**, 178–182.
9. Delagrave, S., Hawtin, R. E., Silva, C. M., Yang, M. M. & Youvan, D. C. (1995) *Biotechnology* **13**, 151–154.
10. Prasher, D. C., Eckenrode, V. K., Ward, W. W., Prendergast, F. G. & Cormier, M. J. (1992) *Gene* **111**, 229–233.
11. Picard, V., Ersdal-Badju, E., Lu, A. & Bock, S. C. (1994) *Nucleic Acids Res.* **22**, 2587–2591.
12. Gossen, M. & Bujard, H. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 5547–5551.
13. Pear, W. S., Nolan, G. P., Scott, M. L. & Baltimore, D. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 8392–8396.
14. Alberti, S., Parks, D. R. & Herzenberg, L. A. (1988) *Cytometry* **8**, 114–119.
15. Moore, W. & Kautz, R. (1986) in *The Handbook of Experimental Immunology*, eds. Weir, D. M., Herzenberg, L. A., Blackwell, C. C. & Herzenberg, L. A. (Blackwell, Edinburgh), pp. 30.1–30.11.
16. Ropp, J. D., Donahue, C. J., Wolfgang-Kimball, D., Hooley, J. J., Chin, J. Y., Hoffman, R. A., Cuthbertson, R. A. & Bauer, K. D. (1995) *Cytometry* **21**, 309–317.
17. Fieck, A., Wyborski, D. L. & Short, J. M. (1992) *Nucleic Acids Res.* **20**, 1785–1791.
18. Rizzuto, R., Brini, M., DeGiorgi, F., Rossi, R., Heim, R., Tsien, R. Y. & Pozzan, T. (1996) *Curr. Biol.* **6**, 183–188.